Impact of hematopoietic growth factors on cancer treatment

Biesma, Bonne

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Stellingen

behorende bij het proefschrift van Bonne Biesma

1. Het positieve effect van rhGM-CSF op het trombocytenaantal in vivo dient niet als een toevalsbevinding te worden afgedaan.

2. Koorts dient als een belangrijke en ongewenste bijwerking van sommige hematopoëtische groeifaktoren beschouwd te worden.

3. Bij patiënten met een solide tumor moeten chemotherapie en een hematopoëtische groeifaktor niet gelijktijdig toegediend worden.

4. Subcutane toediening van een hematopoëtische groeifaktor verdient de voorkeur boven intraveneuze toediening.

5. Om een additioneel anti-tumor effekt van hematopoëtische groeifaktoren naast een effekt van chemotherapie te demonstreren is een meta-analyse noodzakelijk.

6. Indien chirurgische behandeling van het gemetastaseerde carcinoïd onmogelijk is dient, bij afwezigheid van symptomen, geen therapie gestart te worden.

7. Bij het inschatten van de kans op het optreden van ernstige bijwerkingen van "ecstasy", is het bepalen van de plasmaspiegel van beperkte waarde.

8. Voor een kaatsende spits moet je in Friesland zijn.

9. Of een fles wijn goed zal ouderen hangt niet zozeer af van het feit of de wijn voldoende tannine en fruit bevat, alswel van het geduld van de bezitter.

10. ..., a particular problem being Risenshein, a LOL in NAD whose bone marrow had been wiped out by our cytotoxic agents and had failed to regenerate blood cells, which meant that she was bound to die. (In: The House of God, Samuel Shem)

Groningen, 15 september 1993

RIJKSUNIVERSITEIT GRONINGEN

Impact of hematopoietic growth factors on cancer treatment

Proefschrift

ter verkrijging van het doctoraat in de Geneeskunde
aan de Rijksuniversiteit Groningen
op gezag van de Rector Magnificus Dr. S.K. Kuipers
in het openbaar te verdedigen op
woensdag 15 september 1993
des namiddags te 4.00 uur

door

Bonne Biesma

geboren op 1 november 1957 te Heerenveen

Promotores: Prof. Dr. N.H. Mulder
Prof. Dr. M.R. Halie

Referenten: Dr. E.G.E. de Vries
Dr. E. Vellenga
Dr. P.H.B. Willemse

Promotiecommissie: Prof. Dr. G.K. van der Hem
Prof. Dr. J.G. Aalders
Prof. Dr. M. Symann

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Lay-out : Bonne Biesma
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VOORWOORD

Het is een onmiskenbaar gegeven dat het voorwoord het meest gelezen onderdeel van een proefschrift is. Even zo zeker is het dat een voorwoord ook het enige onderdeel van het proefschrift is dat door een ieder begrepen wordt. Dit soort bemerkingen maakt ook dat een promovendus enige bescheidenheid past waar het gaat om het inschatten van de importantie van zijn levenswerk. Niet tegenstaande dat ben ik tevreden met het boekje dat nu voor U ligt.

Dit proefschrift is het resultaat van de samenwerking tussen de Werkgroep Interne Oncologie en de sectie Hematologie van de Vakgroep Inwendige Geneeskunde van de Medische Faculteit te Groningen.

Mijn promotor Prof. Dr. Nanno Mulder wil ik bedanken voor de vele punten op de i in mijn epistels, terwijl ik mijn tweede promotor Prof. Dr. M.R. Halie dank verschuldigd ben voor het beoordelen van mijn proefschrift. Mijn referenten ben ik ook in hoge mate dank verschuldigd. Dr. Liesbeth de Vries was zelden weg te denken uit mijn dagelijkse bestaan. Daardoor ook was het mij gegund om de ontwikkeling van Josée en Esther Kleibeuker tot volwaardige telefoonbeantwoordsters van dichtbij mee te maken. Dr. Edo Vellenga was op veel momenten het rustpunt in de roerige oncologische zee. Ongetwijfeld heeft zijn Friese afkomst daarin een rol gespeeld. De klinische blik van Dr. Pax Willemse was voor mij van grote waarde bij het behandelen van vele patiënten. Binnen de onderzoeksgroep kwam hij frequent met ongedachte oplossingen voor netelige problemen.

Dr. Dirk Sleijfer dank ik voor het ongekend nauwkeurig doorlezen van de door mij geschreven stukken. Daarnaast heb ik veel geleerd van zijn pragmatische benadering van de geneeskunde.

Mijn opleider Prof. Dr. Piet Postmus (Afdeling Longziekten, VU Ziekenhuis, Amsterdam) dank ik enerzijds voor het in mij gestelde vertrouwen en anderzijds voor de zeer plezierige samenwerking waar het de behandeling van patiënten met longkanker in het kader van mijn onderzoek betrof.

Het in vitro deel van dit proefschrift had niet bestaan zonder Dr. Coby Meijer, Hetty Timmer-Bosscha, Mariet Esselink, Phuong Le en Gert Jan Meersma. Ik hoop dat ze mijn pogingen om in ieder geval ongewenste infecties in het laboratorium te voorkomen op waarde hebben geschat.

Dr. Piet Limburg en Johan Bijzet van de sectie Rheumatologie leverden een belangrijke bijdrage in het onderzoek naar de plasmaspiegels van acute fase eiwitten in de behandelde patiënten. De afdeling Nucleaire Geneeskunde (Hoofd: Dr. D.A. Piers) was behulpzaam bij de bestudering van het Capillary Leak Syndrome en TNF-α plasma spiegels.

Dr. W.J. Sluiter van de sectie Endocrinologie speelde een significante rol met betrekking tot de statistische evaluatie van de data.

Jacob Pleiter, Jan Brouwer en Ale Schuurmans zorgden voor de grafische ondersteuning van het geschrevene, terwijl Willy Bruins-van der Wey ervoor zorgde dat een ingezonden artikel al niet bij voorbaat werd geweigerd.

De leden van de promotiecommissie, Prof. Dr. G.K. van der Hem, Prof. Dr. J.G. Aalders en Prof. Dr. M. Symann, ben ik zeer erkentelijk voor het beoordelen van proefschrift.

Wat betreft de geestelijke en lichamelijke ondersteuning van de patiënten sta ik vooral in het krijt bij de oncologie verpleegkundigen Brechtje Oosterhuis en Jos Dijkstra, alsmede bij al het personeel van "Ingang 28" en van de verpleegafdelingen Oncologische Gynaecologie en B3, B1, A1 van de Kliniek voor Inwendige Geneeskunde.

Het verhaal wil dat de militaire diensttijd veelal de mooiste periode is uit het leven van de man. Door een gelukkige samenloop van omstandigheden moest ik deze tijd aan mij voorbij laten gaan. Het doet mij groot genoegen dat deze lacune meer dan opgevuld werd door mijn kamergenoten en medeonderzoekers Dr. Egbert Smit, Drs. Afien Nanninga, Drs. Annemiek Cats, Drs. Jourik Gietema, Drs. Jan Buter, Drs. Mieke van Gameren en Dr. Harm Sinnige. Ik hoop dat ik ze ook in de toekomst niet geheel uit het oog zal verliezen.

Op deze plaats zou ik eigenlijk Janny moeten bedanken voor de extra steun die zij mij gaf in de afgelopen periode. Echter, het lukte mij niet om een concreet voorbeeld voor de geest te halen. Dit ondersteunt dus volledig de mening van al onze vrienden: ik werd al veel langer in de watten gelegd.

CONTENTS

INTRODUCTION

Hematopoietic growth factors are glycoproteins that play an important role in the regulation of the hematopoiesis by affecting the proliferation and differentiation of hematopoietic progenitor cells (1).

One of the major side effects of chemotherapy treatment is myelosuppression, which apparently cannot be prevented by endogenous production of hematopoietic growth factors. The risk of infectious complications is closely related to the severity and duration of the chemotherapy-related neutropenia (2), while severe thrombocytopenia increases the risk of hemorrhagic complications. Myelosuppression very often also is the limiting factor in attempts to increase the chemotherapy dose administered to patients. Chemotherapy dose-intensification may be an important tool in increasing response and survival rates in cancer patients (3, 4).

With the cloning of several hematopoietic growth factors (5-7), such as Granulocyte-macrophage Colony-stimulating Factor (GM-CSF), Granulocyte (G)-CSF, and Interleukin-3 (IL-3), the opportunity has been created to test in vivo whether exogenously applied recombinant human (rh) hematopoietic growth factors can sufficiently ameliorate chemotherapy-induced myelosuppression or enhance bone marrow restoration after chemotherapy, thus reducing the risk of hemorrhagic and infectious complications.

If such is the case then, with hematopoietic growth factor-support, attempts could be made to intensify chemotherapy treatment without increasing the risk for the complications mentioned above.

The main goal of this thesis was to study whether application of the hematopoietic growth factors rhGM-CSF and rhIL-3 after chemotherapy can reduce the severity of neutropenia and thrombocytopenia at the cost of few growth factor-related side effects.

Chapter 1 reports on the effects of several hematopoietic growth factors on the in vitro proliferation of small cell lung cancer cell lines. This in view of potentially hazardous effects of these growth factors when administered to patients with cancer.

Chapter 2 contains the results of a study in which rhGM-CSF was administered to patients with chemotherapy-related leukopenia and fever. The primary goal of this trial was to study whether this patient group could potentially benefit from rhGM-CSF treatment, leading to a reduction in the duration of neutropenia. This may lead to a reduced duration of morbidity, antibiotic use, hospitalization, and subsequently in a reduction in health care costs.

Chapters 3 and 4 provide the results of phase I/II trials with rhGM-CSF and rhIL-3, respectively, administered after chemotherapy to patients with ovarian cancer. As outlined above, the emphasis in these studies was on the effect of these growth factors on the severity of myelosuppression.

In chapter 5 the results of a pharmacokinetic analysis of rhIL-3 administered to ovarian cancer patients treated with chemotherapy, as described in chapter 4, are reported.

Chapter 6 consists of a review on the effects of hematopoietic growth factors on chemotherapy-induced myelosuppression.

In chapter 7 the results of our studies are summarized and the state of the art concerning hematopoietic growth factors is briefly discussed.

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Chapter 1

THE EFFECTS OF FIVE HEMATOPOIETIC GROWTH FACTORS ON HUMAN SMALL CELL LUNG CARCINOMA CELL LINES: INTERLEUKIN-3 ENHANCES THE PROLIFERATION IN ONE OF THE ELEVEN CELL LINES

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SUMMARY

Eleven small cell lung carcinoma cell (SCLC) lines of human origin were exposed to different colony stimulating factors (CSFs), to study whether CSFs could enhance the spontaneous cell proliferation and modify the action of cytotoxic drugs. In ten cell lines no suppressive or stimulative effect was observed, when measured in a ^{3}H-thymidine assay and MTT assay. However, one cell line (GLC-20) could be stimulated by interleukin-3 (IL-3) when measured with a proliferative as well as clonogenic assay. This enhancing effect was cell concentration dependent in the ^{3}H- thymidine assay. Additional CSFs such as Granulocyte-Macrophage (GM)-CSF, Granulocyte (G)-CSF, IL-4, IL-6, insulin, or bombesin could not further augment the IL-3 supported proliferation. In addition, IL-3 binding studies demonstrated the presence of IL-3 receptors on the GLC-20 cells. Two types of receptors were demonstrated by Scatchard analysis: high affinity receptors (59 $\pm$ 4 sites per cell) with a dissociation constant (Kd) of 31 $\pm$ 9 pmol/l, and low affinity receptors (1915 $\pm$ 91 sites per cell) with a Kd of 2.0 $\pm$ 0.8 nmol/l. Finally, it was shown that the toxic effects of adriamycin and cisplatin on the proliferation of the GLC-20 cell line could partially be abrogated in the presence of IL-3. These data indicate that in some cases CSFs can modulate the proliferation of SCLC cell lines and interfere with the effects of chemotherapeutic drugs.

INTRODUCTION

Granulocyte-Macrophage colony stimulating factor (GM-CSF), granulocyte (G)-CSF, and interleukin-3 (IL-3) are glycoproteins that stimulate the proliferation and differentiation of

normal and leukemic hematopoietic progenitors in vitro as well as in vivo (1-3). The genes of these factors have been cloned resulting in large amounts of CSFs being available. This has provided the opportunity to modulate in vivo hematopoiesis during periods of chemotherapy induced bone marrow hypoplasia (4, 5). However, the action of the CSFs is not restricted only to hematopoietic cells but can also affect the proliferation of non-hematopoietic cells (6-10). Studies have shown that in vitro these growth factors can stimulate or inhibit growth of various non-hematopoietic tumor cells. Their effects with regards to small cell lung carcinoma cell lines (SCLC) remain controversial; one study has shown anti-proliferation (6) but Munker et al. (7) have observed no effects, although recent studies provided evidence for the presence of GM-CSF and G-CSF receptors on SCLC cell lines (11,12).

In view of the increasing clinical application of CSFs in solid tumors, we have studied the effects of five CSFs (GM-CSF, G-CSF, IL-3, IL-4, and IL-6) on a panel of eleven human SCLC cell lines, focusing attention on the capability of the CSFs to affect the proliferation of these malignant cells and to modulate the effects of chemotherapeutic agents.

MATERIALS AND METHODS

Materials

Recombinant human IL-3, G-CSF, and GM-CSF were gifts from Dr. S.C. Clark, Genetics Institute, Cambridge. Recombinant human IL-4 was a gift of Dr. P. Trotta, Schering Corporation, Bloomfield, New Jersey. Recombinant IL-6 was a gift from Dr. L.A. Aarden, CLB, Amsterdam, The Netherlands. The concentrations used in this study were based on the response of myeloid and erythroid progenitors to the different CSFs as shown earlier by Vellenga et al. (2, 3, 20). In the case of no response, five fold higher concentration was used in the different assays, except for G-CSF. This CSF was used at a dilution of 1:1000 and 1:500.

Insulin, bombesin and 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyltetrazoliumbromide (MTT) were purchased from Sigma. Cisplatin (CDDP) was obtained from Bristol Myers S.A.E., Madrid, Spain, and adriamycin from Farmitalia Carlo Erba, Milan, Italy. ^{125}I-IL-3 was purchased from Amersham Laboratories, Amersham, England.

Cell lines

Eleven human small cell lung carcinoma cell lines were used in the experiments: Groningen Lung Carcinoma (GLC)-1, GLC-2, GLC-3, GLC-4; a CDDP resistant subline (GLC4-CDDP) (13); an adriamycin resistant subline (GLC4-Adr) (14); GLC-8 and three cell lines from one patient obtained before and during clinical follow-up GLC-14, GLC-16, GLC-19 (15), and GLC-20. The cell lines were maintained in an atmosphere of 5% CO_2 at 37°C and cultured in Roswell Park Memorial Institute (RPMI) 1640 medium (Gibco, Paisley, Scotland) with 10%

heat inactivated fetal bovine serum (FBS, Hyclone, Logan, UT). The characteristics of the different SCLC cell lines are reported in table 1.

Table 1. SCLC cell line characteristics

Cell line	Sex	Type	Derived from	Culture doubling time (h)	Previous therapy
GLC-1	M	v	Pleural effusion	21	CT
GLC-2	M	v	Pleural effusion	37	-
GLC-3	M	v	Pleural effusion	20	CT
GLC-4	M	v	Pleural effusion	15	CT
GLC-4-CDDP[a]	M	v	Pleural effusion	24	CT
GLC-4-Adr[b]	M	v	Pleural effusion	18	CT
GLC-8	M	c	Primary tumor	34	CT
GLC-14[c]	F	c	Lymph node	26	-
GLC-16[c]	F	c	Pleural effusion	27	CT
GLC-19[c]	F	v	Tumor (relapse)	44	CT+RT
GLC-20	M	v	Primary tumor	49	CT

[a]: In vitro acquired cisplatin resistant subline from GLC-4. [b]: In vitro acquired Adriamycin resistant subline from GLC-4. [c]: Three cell lines derived from one patient during clinical follow-up.
M: male, F: female, v: variant or c: classical cell line, CT: chemotherapy, RT: radiotherapy, - : no previous therapy.

Proliferation assays

DNA synthesis was assessed in a ^{3}H-thymidine proliferation assay. Cells were cultured in 150 μl RPMI 1640 medium with 10% FBS in 96-well round-bottom microtiter plates (Greiner, West Germany) in triplicate. In some experiments the cells were cultured with a lower concentration of FBS (3%); in these cases RPMI 1640 medium was replaced by $SCLC_2$ medium (16). After 4 days of culture (cell concentration of 10^3-10^5), 0.1 μCi of ^{3}H-thymidine with specific activity of 2 Ci/mmol was added to each well, 6 h prior to cell harvest. Radioactivity was determined by liquid scintillation counting. In the experiments the CSF was added at the initiation of the culture.

The effects of chemotherapeutic drugs on the spontaneous proliferation of the GLC-20 cell line were studied in the ^{3}H-thymidine assay. In these experiments adriamycin (0.25, 0.50, 1.0 nM) and cisplatin (0.4 μM) were added at the start of the culture in the presence or absence of IL-3.

The microtiter well tetrazolium assay (MTT assay) is based on the cellular reduction of MTT by mitochondrial dehydrogenase of viable cells to a blue formazan product which can be measured spectrophotometrically (17, 18). Before the assays were performed a linear relationship of cell number to MTT formazan crystal formation was checked. The relationship between seeding density, incubation volume and incubation period was established for each cell line separately after growth studies. Incubation of 5,000, 12,500, 15,000, 35,000, 10,000 and 30,000 cells per well for GLC-4, GLC-4 Adr, GLC-4 CDDP, GLC-14, GLC-16, GLC-19, GLC-3, and GLC-20 respectively, proceeded in a total volume of 0.1 ml with and without CSFs (for GLC-3 and GLC-20 a total volume of 0.25 ml was used) in 96-well culture plates (NUNC, Gibco, Paisley, Scotland). After a culture period of 4 days (GLC-4, GLC4-Adr, GLC4-CDDP, GLC-14, GLC-16, GLC-19), 5 days (GLC-3) or 11 days (GLC-20), 20 μl of MTT solution (5 mg MTT/ml PBS) was added to each well for 225 min. Thereafter, plates were centrifuged (15 min, 180 g) and the supernatant was carefully aspirated. Dimethyl sulfoxide 100% (200 μl, Merck, Darmstadt, West-Germany) was used to dissolve the formazan crystals and the plates were read immediately at 520 nm using a scanning microtiter well spectrophotometer (Titertek Multiskan, Flow Lab, Irvine, Great Britain). The percentage cell survival was calculated by the formula: mean of the test samples/mean of three untreated samples. Controls consisted of media without cells (background extinction) and cells incubated in wells with medium only.

Soft agar clonogenic assay

The soft agar clonogenic assay (19) was utilized for the GLC-20 cell line. Cells were plated at 5,000 cells/ml in an upper layer of 0.3% agarose in Dulbecco Modified Eagle/F12 (1:1) medium and 20% FBS over an underlayer of 0.5% agar in DME/F12, 20% FBS in a 35 mm Petri dish. Colonies (> 40 cells) were counted on day 20 with an inverted microscope. The IL-3 was added in the upper layer at the initiation of the culture.

IL-3 binding assay

The specific radioactivity of the labeled IL-3 was 480 Ci/mmol and the biological activity was greater than 95 percent of the unlabeled cytokine. The sample has been shown as a single band at Mr 15,000 on gel electrophoresis. ^{125}I-IL-3 was stored at -20°C and the experiments were conducted within 4 weeks after receiving the labeled batch. Cells were washed twice in Hanks' balanced salt solution (Gibco). Usually, 1.5 to 2.5 x 10^6 cells were incubated for one h at 37°C in 100 μl minimal essential medium (Gibco)/10% FBS containing 25 mmol/l HEPES with 10 to 4,000 pM/l radiolabeled IL-3 with or without 100 fold excess of unlabeled IL-3. The cells were then cooled to 0°C, layered over 500 μl FBS in Eppendorf tubes on ice and centrifuged for five minutes at 1,000 g for 5 min. The tubes were frozen in liquid nitrogen, and the tips were cut off for counting in a SRL-4 counter. Specific binding was defined as the difference between the amount of radioactivity bound without unlabeled factor added, and the amount of

radioactivity bound with excess unlabeled factor added. Pilot experiments demonstrated that specific binding at 37°C proceeded rapidly and reached a plateau after one hour incubation. All experiments were conducted in duplicates. Scatchard analysis was performed with the Elseviers Enzfitter program by using the two binding place model as well as the one binding place model. The Scatchard plot obtaining curve indicated the presence of two binding sites. In all cases the two receptor model was preferred. Statistical tests for discriminating between one and two receptor models were performed with Statgraph statistical program.

Statistical analysis

The paired Student's t-test was used for statistical analysis. P values < 0.05 were considered significant.

RESULTS

The proliferative response of eleven SCLC cell lines was tested in at least three different experiments in the presence of different concentrations of GM-CSF (10, 50 ng/ml), G-CSF (1:1000, 1:500), IL-3 (100, 500 U/ml), IL-4 (10, 50 ng/ml), and IL-6 (100, 500 U/ml). DNA synthesis measured in ^{3}H-thymidine assay and the survival of cells measured in the MTT test, could not be modified by the different CSFs in ten SCLC cell lines; there was no suppression or stimulation. In addition, no difference was noted in the presence of different cell concentrations, 10^3 to 10^5 cells.

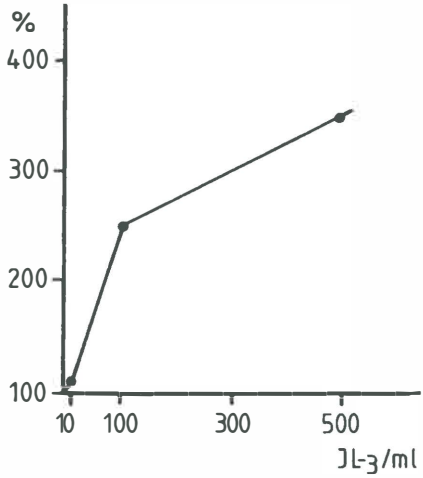

Figure 1. Proliferative response of the GLC-20 cell line in the presence of variable concentrations of IL-3. Different concentrations of IL-3 were added to $1x10^5$ cells/150 μl. The % increase reflects the ratio of the ^{3}H-thymidine uptake in the presence or absence of IL-3 x100%. Values represent the mean of triplicate cultures. 1 SD was 5% to 15% of the mean.

Table 2. Effects of 5 CSFs on the proliferation of the GLC-20 cell line

Stimulant	^{3}H-Thymidine incorporation (DPS)
None	228 ± 13
IL-3	452 ± 12
G-CSF	217 ± 9
GM-CSF	223 ± 8
IL-4	207 ± 11
IL-6	240 ± 10
IL-3 + G-CSF	390 ± 24
IL-3 + GM-CSF	437 ± 30
IL-3 + IL-6	379 ± 6

Different CSFs or combinations were added to 10^5 cells/150 μl during 4 days of culture. The following concentrations or dilutions were used: IL-3 300 U/ml; G-CSF 1:1000; GM-CSF 30 ng/ml; IL-4 300 U/ml; IL-6 100 U/ml. Results are mean disintegration per second (DPS) ± SD of triplicate cultures. A significant difference ($p < 0.001$) was observed between the IL-3 supported proliferation versus control.

Table 3. The effects of bombesin and insulin on the proliferation of the GLC-20 cell line in the ^{3}H-thymidine assay

	^{3}H-Thymidine uptake (DPS)			
	1 x 10^4		1 x 10^5	
	- IL-3	+ IL-3	- IL-3	+ IL-3
Control	113 ± 5	132 ± 7	192 ± 21	373 ± 28
Bombesin	118 ± 4	122 ± 21	173 ± 4	406 ± 31
Insulin	119 ± 4	136 ± 4	177 ± 10	396 ± 22

IL-3 (300 U/ml), insulin (10 μg/ml), and bombesin (50 nM) were added to 10^4 or 10^5 cells/150 μl during four days of culture. Results are mean disintegration per second (DPS) ± SD of triplicate cultures. A significant difference was noted at a cell concentration of 10^5 cultured in the presence or absence of IL-3 ($p < 0.001$).

However, different results were obtained with the GLC-20 cell line. In the ^{3}H-thymidine assay IL-3 (300 U/ml) enhanced the spontaneous cell proliferation at a cell concentration of 10^5 cells/150 μl (212 $\pm$ 18 versus 395 $\pm$ 50 DPS, x $\pm$ SD, N=3, $p < 0.001$). No effect was observed at a lower cell concentration (10^3-10^4 cells). A dose-response curve with IL-3 demonstrated a dose-dependent increase in DNA synthesis (fig. 1). This stimulatory effect of IL-3 could not be further enhanced by an additional growth factor when the IL-3 concentration remained 300 U/ml (table 2). The combinations of GM-CSF and IL-3, G-CSF and IL-3, and IL-6 and IL-3 demonstrated an increase of 166%, 171%, and 191% respectively, while an increase of 198% was observed in the presence of IL-3 alone. Furthermore, it appeared that the proliferative response was independent of the FBS concentration. In the presence of 10% FBS, an increase in the proliferative response was observed of 340% in the presence of 500 U/ml IL-3, while in the presence of 3% FBS the proliferation was enhanced by 320%, although in these experiments a more enriched medium ($SCLC_2$) was used. The observed increase in thymidine uptake reflected DNA synthesis since comparable results were observed in the clonogenic assay. In the absence of IL-3 800 $\pm$ 47 colonies/5 x 10^3 cells were detected after 20 days of culture, while the colony number increased to 1060 $\pm$ 127 in the presence of 100 U IL-3 /ml. Moreover, the colony size increased in the presence of IL-3.

The enhancing effects of IL-3 on the spontaneous cell proliferation of the GLC-20 was not observed in the MTT assay since in this assay cell concentrations of $3x10^4$ cells/250 μl were used. However, by using a higher cell concentration (10^5 cells/150 μl) in this assay a growth advantage was noticed in the presence of IL-3 in comparison with the control.

Since the cell concentration dependent effect might imply the presence of an additional growth factor released by cell-cell interaction and subsequently not observed at lower cell concentrations, we studied the effects of insulin and bombesin in the presence of IL-3 at a cell concentration of 1-10 $x10^4$/150 μl. As depicted in table 3, IL-3 enhanced the proliferation of the GLC-20 cell line at a cell concentration of 10^5/150 μl, but no further augmentation was noticed by the addition of bombesin (50 nM) or insulin (10 μg/ml). In addition, bombesin and insulin could not abolish the IL-3 unresponsiveness at a lower cell concentration. Comparable results were obtained by the addition of cell-free supernatant of the GLC-20 cell line collected after 2 days of culture (data not shown). Furthermore IL-3 binding assays were performed with ^{125}I-IL-3 to study the IL-3 receptor expression on the cell surface of the GLC-20 cell line. By Scatchard plot analysis two types of receptors could be demonstrated. In two separated experiments high affinity receptors (56 $\pm$ 14 and 62 $\pm$ 28 sites per cell) with a dissociation constant (Kd) of 38 $\pm$ 11 and 25 $\pm$ 6 pmol/l, and low affinity receptors (1850 $\pm$ 360 and 1980 $\pm$ 160 sites per cell) with a Kd of 1.4 $\pm$ 0.2 and 2.6 $\pm$ 0.2 nmol/l were shown. A representative experiment is demonstrated in fig. 2.

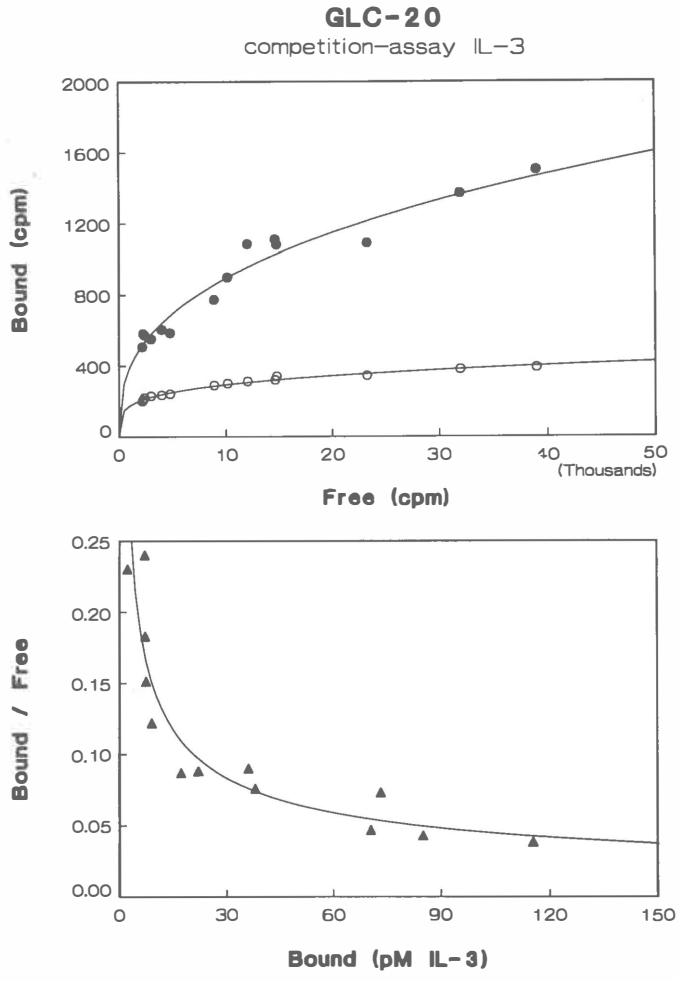

Figure 2. Binding of radiolabeled IL-3 to GLC-20 cells. Cells were incubated with increasing amounts of radiolabeled IL-3 for one h at 37°C. Non specific binding was determined in the presence of excess unlabeled factor. Specific binding (●) as well as nonspecific binding (○) data are plotted (upper panel). Scatchard plot of these data (lower panel). Points reflect averages of duplicate determinations.

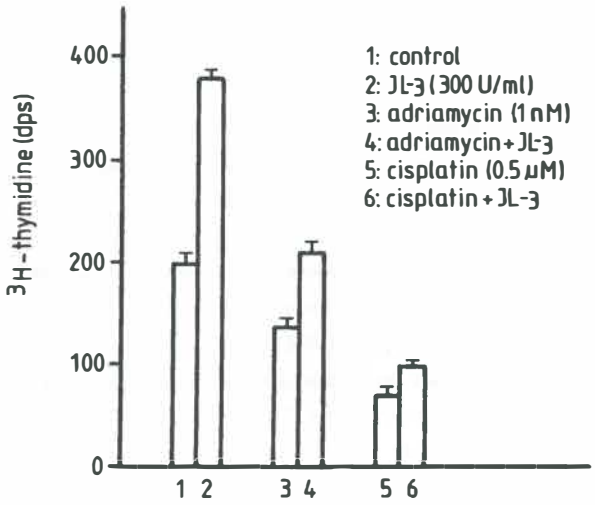

Figure 3. Effects of adriamycin and CDDP on the IL-3 supported proliferation of the GLC-20 cell line.

Finally we tested whether this CSF influenced the effects of two chemotherapeutic drugs on the proliferative capacity of this cell line at different concentrations. A representative experiment is shown in figure 3; adriamycin (1 nM) and CDDP (0.5 μM) reduced DNA synthesis by 32% and 64% respectively. This cytotoxic effect could partially be abrogated by the continuous presence of IL-3 in the cell culture medium.

DISCUSSION

Colony stimulating factors are important regulatory proteins which can stimulate the normal hematopoietic progenitor cell at different levels of differentiation (1). In view of these activities, CSFs are increasingly clinically applied to treat or to circumvent chemotherapy induced aplasia (4, 5). However, the present study demonstrates that the application of these factors may have certain disadvantages.

In one out of eleven SCLC cell lines we observed a stimulatory activity of IL-3 on the spontaneous cell proliferation measured with a ^{3}H-thymidine uptake and colony assay. This effect was dose-, and cell concentration dependent. The cell concentration dependent effect of IL-3 might indicate the presence of a co-stimulatory factor, secreted by the SCLC cells through cell-cell interaction and acting in the presence of IL-3. However, insulin and bombesin, the most prominent autocrine growth factors for SCLC cells (21, 22), or supernatants could not mimic these effects in the ^{3}H-thymidine assay at lower cell concentrations. These data might indicate that a undefined protein, secreted by the SCLC cell line is required for the action of

IL-3, or that cell-cell interaction modifies the responsiveness of cells for the action of CSFs. Rizzino et al. (23) observed that cell density influenced the growth factor receptor expression, the so-called density-induced down regulation. High cell density resulted in a decrease in the binding of growth factors, indicating that cell-cell interaction might regulate the response to growth factors as a result of changes in the receptor expression. It is conceivable that comparable mechanisms may operate in the GLC-20 cell line whereby the results with the clonogenic assay might be ascribed to the fact that IL-3 acts on the tightly attached cells in the clonogenic assay as result of the spontaneous colony formation. In addition the results indicate that there is a direct effect of IL-3 on the clonogenic progenitor cell.

The IL-3 bindings studies demonstrate the presence of IL-3 receptors on the cell surface of the GLC-20 cell line and explain the stimulatory activity of IL-3 in the ^{3}H-thymidine and clonogenic assay. The receptor numbers and dissociation constant corresponds with results obtained with hematopoietic cells (24).

The experiments concerning the effects of adriamycin and cisplatin on the IL-3 supported proliferation demonstrate that the continuous presence of IL-3 can partially abrogate the cytostatic effects of these agents. This observation may have clinical impact since different clinical trials, e.g in acute myeloid leukemia, explore the possibility to apply a CSF before or during chemotherapy focused to synchronize cells in the cell cycle. Further studies will be necessary to study interactions between hematopoietic growth factors and chemotherapeutic drugs. Changes in time schedules for CSFs and chemotherapy may influence results in vitro and in the clinic. In view of the observed effects this may also apply to solid tumors.

In summary, these data demonstrate that in a minority of cases CSFs can modulate the spontaneous proliferation of malignant SCLC cells. This might have clinically consequences since CSFs have the capacity to interfere with the effects of chemotherapeutic drugs.

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Chapter 2

EFFICACY AND TOLERABILITY OF RECOMBINANT HUMAN GRANULOCYTE-MACROPHAGE COLONY-STIMULATING FACTOR IN PATIENTS WITH CHEMOTHERAPY-RELATED LEUKOPENIA AND FEVER

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SUMMARY

Thirty patients with chemotherapy related leukopenia ($\leq$ 1.0 x10^9/l) and fever ($\geq$ 38.5 ^{0}C) were treated in a double-blind placebo controlled trial with standard antibiotics and seven days of intravenously administered recombinant human Granulocyte-Macrophage Colony-Stimulating Factor (GM-CSF) or placebo (2.8 μg/kg/day). GM-CSF administration resulted in a faster percentage increase of peripheral neutrophil count after two and three days of treatment, except in patients treated with ablative chemotherapy and autologous bone marrow transplantation. However, GM-CSF did not shorten the period of fever or antibiotic administration. No side effects were observed, especially no increased release of cytokines was noticed, such as Tumor Necrosis Factor-alpha and Interleukin-6. These data suggest that a subpopulation of patients with chemotherapy related leukopenia and fever may benefit from GM-CSF treatment in view of the observed effects on neutrophil count. An extended study is needed to evaluate whether this implies a reduction in antibiotic treatment and morbidity.

INTRODUCTION

Recently, several colony stimulating factors (CSF's) have been cloned (1-3), which offered the opportunity to test in vivo whether CSF's can circumvent chemotherapy-related leukopenia or accelerate bone marrow restoration after treatment with chemotherapeutic agents (4-12). Such an effect might decrease the risk of infections in patients with chemotherapy-related leukopenia, especially in patients with a granulocyte count below 0.5x10^9/l (13, 14).

Apart from stimulating the in vitro and in vivo proliferation of immature myeloid

progenitor cells (15, 16), Granulocyte-Macrophage CSF (GM-CSF) also enhances the functional activities of neutrophils and monocytes such as superoxide production (17-21), phagocytic activity (18, 22-24) and cytokine release (24-26). The induction of cytokines by GM-CSF may have certain disadvantages during a period of septicaemia, as in vitro studies have shown that GM-CSF can enhance the endotoxin-induced TNF release from monocytes (26).

In the present double-blind randomized trial we studied whether GM-CSF enhanced bone marrow restoration during a period of leukopenia and fever following chemotherapy. In addition we measured Tumor Necrosis Factor-alpha (TNF-α) and Interleukin-6 (IL-6) serum levels in five patients with gram-positive septicaemia during the first hours of GM-CSF administration.

MATERIALS AND METHODS

Study design

Thirty consecutive patients, between 18 and 75 years of age, with fever ($\geq$ 38.5°C, measured by Terumo digital clinical thermometer for axillary use) and chemotherapy-related leukopenia ($\leq$ $1.0x10^9$/l) were entered in the study. Patients already treated with antibiotics were not entered in the study. All patients gave informed consent. Patients with severe heart, lung, liver (serum total bilirubin $\geq$ 80 μmol/l) or kidney impairment (creatinine clearance $\leq$ 30 ml/min) were excluded from the study, as were patients with acute myeloid leukemia or refractory anemia. The study was a double-blind randomized trial with fifteen patients receiving GM-CSF and fifteen patients receiving placebo. GM-CSF or placebo was administered as a continuous intravenous infusion for 7 days and was started simultaneously with intravenous antibiotic therapy. Standard antibiotic treatment consisted of tobramycin (3 x 1.5 mg/kg/day if creatinine clearance $>$ 100 ml/min.) and cefuroxime (3 x 1.5 g/day) except in patients treated with autologous bone marrow transplantation (ABMT). These patients were treated with netilmycin (3 x 2 mg/kg/day) and cefuroxime (3 x 1.5 g/day). The duration of antibiotic administration was primarily based on the body temperature of the patient. Antibiotic treatment was not discontinued unless the body temperature was normal for at least two days. The study was approved by the Medical Ethical Committee of the University Hospital Groningen.

Recombinant human glycosylated mammalian derived GM-CSF, with a specific activity of $8x10^6$ U/mg protein, was provided by Sandoz/Schering Plough (Basle, Switzerland). The vials contained 0.216 mg glycosylated GM-CSF after reconstitution with the enclosed vehicle. Lyophilized placebo in identical vials was also supplied by Sandoz/Schering Plough. The dosage of GM-CSF was 2.8 μg protein/kg/day by continuous intravenous infusion for seven days. The daily dosage of either active drug or placebo was dissolved in 500 ml glucose 5%. Intravenous administration of GM-CSF or placebo was discontinued if thrombophlebitis developed. The drug was then given subcutaneously twice daily, or when a shortage of infusion

Table 1. Patient characteristics

	GM-CSF group	Placebo group
Male/female	9/6	7/8
Median age in years (range)	44 (21-74)	52 (18-66)
Diagnosis (no. of patients)	Breast carcinoma (5)	Breast carcinoma (6)
	Small cell lung carcinoma (4)	Small cell lung carcinoma (5)
	Non small cell lung carcinoma (1)	Non-Hodgkin's lymphoma (3)
	Burkitt's lymphoma (1)	Neuroblastoma (1)
	Bladder carcinoma (1)	
	Gastric carcinoma (1)	
	Non-seminomatous germ cell carcinoma (1)	
	Acute lymphoblastic leukemia (1)	
Previous radiotherapy	4	1

possibilities occurred.

Blood counts, including differential counts and reticulocyte counts, were performed at entry, daily during GM-CSF or placebo administration, on the two days following GM-CSF or placebo administration, and twice weekly during the two weeks thereafter. Liver and renal functions were determined at entry, three times during drug administration, and once weekly in the following two weeks. At the same time points serum levels of cholesterol, sodium, potassium, calcium, total protein and albumin were measured.

TNF-α and IL-6 levels

TNF-α and IL-6 serum levels were retrospectively determined before, and one and four hours after the start of GM-CSF administration in five patients with gram-positive septicaemia. TNF-α (normal value < 5 ng/l) was measured with a radio-immunoassay (Medgenix, Brussels, Belgium). IL-6 (normal value < 20 U/ml) was detected with the B9 bioassay (27).

Statistical analysis

Student's t test, the chi-square test for small numbers according to Yates, the Wilcoxon rank sum test and the Sign test on rank differences were used for statistical analysis. Only p-values < 0.05 were considered significant.

RESULTS

Patient characteristics

Patient characteristics are shown in table 1. The median age was 44 years (range 21-74) in the GM-CSF group and 52 (range 18-66) in the placebo group. The histology of the primary tumors is reported in table 1. Eleven patients were treated for breast carcinoma (GM-CSF group 5; placebo group 6) and nine patients for small-cell lung carcinoma (SCLC, GM-CSF group 4; placebo group 5). Five patients in the GM-CSF group (four with breast carcinoma and one with Burkitt's lymphoma) and three patients in the placebo group (two with breast carcinoma and one with non-Hodgkin's lymphoma) had received ablative chemotherapy with ABMT. Five patients had received prior radiotherapy. All patients had a leukocyte count > 3.0×10^9/l and platelet count > 100×10^9/l at the start of the last chemotherapy course.

Table 2. Clinical characteristics[a]

	GM-CSF group	Placebo group
Days since last chemotherapy course	8.5±3.9[b] (n=15)	6.7±1.4 (n=15)
Duration of temperature ≥ 38.0°C (days) after start of GM-CSF or placebo	2.9±3.6 (n=12)	2.4±2.5 (n=12)
Infections		
Positive blood culture	7	3
Gram-negative	1	-
Gram-positive	6	3
Other positive cultures	1	1
Urinary tract	1	-
Rectovaginal septum	-	1
Clinically documented infections	2	4
Throat	2	2
Sinus	-	2
Fever of unknown origin	5	7

[a]: No significant differences between the groups, [b]: Mean ± SD.

The clinical characteristics of the studied group are reported in table 2. The time since the last chemotherapy course was 8.5 ± 4.0 (mean±SD) days in the GM-CSF group and 6.7 ± 1.4 days in the placebo group (NS). In the GM-CSF group positive blood cultures were documented at the start of the study in 47% of the patients. In the placebo group 20% of the patients had a positive blood culture. Other documented infections at the start of the study were: a urinary tract infection with Escherichia Coli in the GM-CSF group; a gram-positive culture of a phlegmon of the recto-vaginal septum in the placebo group. In addition, two clinically documented infections were observed in the GM-CSF group and four in the placebo group (table 2).

Hematological recovery of the patients treated with standard dose chemotherapy

At the start of the study the mean leukocyte counts demonstrated no significant difference between both groups: GM-CSF group 0.5 ± 0.3x10^9/l (mean±SD), n=9, placebo group 0.6 ± 0.3x10^9/l, n=11 (Table 3). In addition, no difference was noticed in the mean neutrophil counts at day 0 (GM-CSF group: 0.07 ± 0.09x10^9/l, n=9; placebo group: 0.11 ± 0.12x10^9/l, n=11). Absolute mean monocyte and eosinophil count at day 0 were 0.1 ± 0.1x10^9/l, and 0.01 ± 0.02x10^9/l, n=9 respectively in the GM-CSF group versus 0.08 ± 0.09x10^9/l, and 0.01 ± 0.02x10^9/l, n=11 in the placebo group (NS).

Table 3. Peripheral leukocyte and neutrophil counts in leukopenic patients treated for seven days with GM-CSF or placebo after standard-dose chemotherapy

	Leukocytes (x 10^9/l)		Neutrophils (x 10^9/l)	
Day	GM-CSF (n=9)	Placebo (n=11)	GM-CSF (n=9)	Placebo (n=11)
0	0.46±0.32	0.64±0.32	0.08±0.10	0.09±0.11
1	0.64±0.49	0.67±0.46	0.15±0.24	0.11±0.12
2	1.39±1.78	0.95±0.65	0.22±0.45	0.25±0.29
3	2.80±3.98	1.74±1.75	2.11±3.70	1.00±1.62
4	4.19±5.62	2.83±3.62	3.21±5.00	0.96±1.18
5	6.78±8.18	3.72±3.36	5.21±7.00	2.24±3.31
6	8.60±10.1	4.83±4.22	6.35±7.90	3.26±4.10
7	11.0±12.0	5.61±4.80	8.60±10.1	3.95±4.67

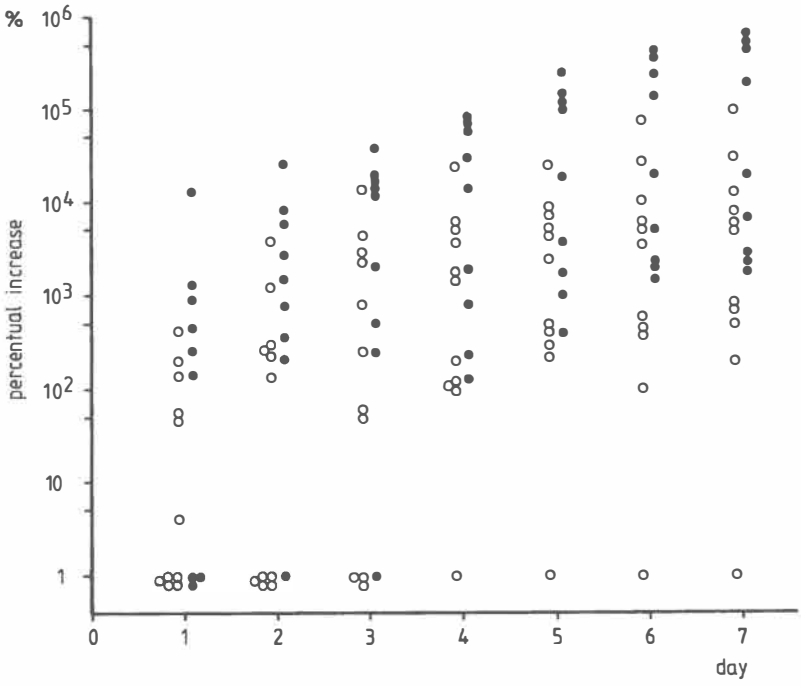

Figure 1. Percentage increase of neutrophils compared with start of study for GM-CSF (●) and placebo treated patients (○) after standard-dose chemotherapy.

After two days of treatment there was a significantly higher percentage increase of the neutrophil count compared with day 0 (fig.1) in the GM-CSF group (4916 ± 8103%, n=9) versus the placebo group (513 ± 1120%, n=11, $p < 0.05$, Wilcoxon rank sum test). However, this difference disappeared after seven days of treatment (GM-CSF group 205447 ± 264785%, n=9; placebo group 14649 ± 29219%, n=11, Wilcoxon rank sum test). Furthermore, the percentage increase of the neutrophils was significantly higher ($p < 0.005$, Sign test on rank differences) during the whole treatment period in the GM-CSF than in the placebo group. The absolute numbers of leukocytes and neutrophils during the treatment period are reported in table 3. Differences in leukocyte and neutrophil counts between the GM-CSF and placebo group were not statistically significant.

After seven days of treatment the absolute numbers of monocytes and eosinophils were respectively 1.0 ± 1.1x10^9/l and 0.13 ± 0.22x10^9/l, n=9 in the GM-CSF group versus 0.75 ± 0.36x10^9/l and 0.01 ± 0.01x10^9/l, n=11 in the placebo group (NS). A neutrophil count > 0.5x10^9/l was reached in the GM-CSF group and the placebo group after 6.3 ± 3.2 (mean±SD) and 6.7 ± 4.3 days (NS), respectively. Three patients reached well above the average leukocyte and neutrophil counts. In the GM-CSF group one patient reached leukocyte

counts of $38.0x10^9$/l (neutrophils $32.0x10^9$/l) after 7 days of treatment. In the placebo group two patients reached leukocyte counts of 13.9 and $15.4x10^9$/l (neutrophils 12.8 and $13.0x10^9$/l) at day 7. All three patients demonstrated signs of persisting infections. The recovery of lymphocytes, basophils, platelets (144 $\pm$ $140x10^9$/l, n=9 in the GM-CSF group and 187 $\pm$ $131x10^9$/l, n=11 in the placebo group at day 7) and reticulocytes was not affected by GM-CSF administration in this group of patients.

Hematological recovery of the patients treated with ablative chemotherapy and ABMT

Mean leukocyte counts at the start of the study demonstrated no significant difference between the two groups: GM-CSF group 0.06 $\pm$ $0.03x10^9$/l (n=5), placebo group 0.08 $\pm$ $0.04x10^9$/l (n=3). Neutrophil counts at entry and after two days of treatment were zero in two patients in the placebo group and in three patients in the GM-CSF group. An additional patient in the GM-CSF group (neutrophil count at entry zero) showed a neutrophil count of $0.02x10^9$/l at day 2. The fifth patient in the GM-CSF group showed a neutrophil count of 0.02 and $0.04x10^9$/l at entry and at day 2, respectively. However, GM-CSF administration in the last two patients was started six days after bone marrow reinfusion, compared with 3.4 $\pm$ 1.8 (mean$\pm$SD) days in the other five patients. Absolute numbers of leukocytes (and neutrophils) after seven days of treatment were 1.5 $\pm$ $0.98x10^9$/l, n=3 (and 0.77 $\pm$ $0.63x10^9$/l, n=3) in the GM-CSF group and 1.1 $\pm$ $1.6x10^9$/l, n=3 (and 0.72 $\pm$ $1.17x10^9$/l, n=3) in the placebo group (NS), respectively. In addition, GM-CSF did not affect the recovery of eosinophils, monocytes, lymphocytes, basophils, platelets and reticulocytes.

Clinical results

The number of days with fever after initiation of treatment was not different between both groups (GM-CSF group 2.9 $\pm$ 3.6 days; placebo group 2.4 $\pm$ 2.5 days, median 1.5 days in both groups). Duration of fever in GM-CSF treated patients after standard dose chemotherapy was 2.7 $\pm$ 3.1 days, n=9 (placebo group 1.8 $\pm$ 2.1 days, n=9), and after ABMT 3.7 $\pm$ 5.5 days, n=3 (placebo group 4.3 $\pm$ 3.2 days, n=3). In the GM-CSF group three patients were not evaluable with regard to the duration of fever because of the sudden death of one patient on the first day of treatment and premature discontinuation of GM-CSF on day 5, while fever still persisted in two patients. In the placebo group two patients were treated with prednisolone from the start of the study and in one patient treatment was discontinued prematurely on day 4. Finally, no significant difference was noticed with regard to the period of antibiotic administration between GM-CSF and placebo treated patients (10.8 $\pm$ 4.4 versus 9.6 $\pm$ 4.2 days). Patients not evaluable for days of fever were also considered not to be evaluable for days of antibiotic treatment.

Side effects

There was no significant difference in the incidence (GM-CSF group 6/11 patients, placebo

group 4/12 patients, chi-square test) and day of onset of thrombophlebitis (GM-CSF group 2.8 ± 1.7 days, placebo group 3.9 ± 1.0 days). In the GM-CSF group the administration of GM-CSF was changed to subcutaneous administration in two patients and in the placebo group in four patients. In both groups one patient received the drug subcutaneously and two patients through a central venous catheter since the start of treatment. No other side effects related to GM-CSF were observed such as fever, myalgia, bone pain or erythema. In none of the patients excessive weight gain as sign of capillary leakage was noticed. GM-CSF did not affect liver and renal functions, nor did it affect cholesterol levels (GM-CSF group: 3.9 ± 0.9 mmol/l (mean±SD) and 3.2 ± 1.0 mmol/l, the placebo group: 3.97 ± 2.1 mmol/l and 4.1 ± 1.7 mmol/l, at entry and day 7, respectively).

In the GM-CSF group three patients did not complete the study. One patient, with gram-positive septicaemia, died within five hours after the start of GM-CSF therapy probably due to a septic shock or cerebral bleeding (permission for autopsy was not obtained). In one ABMT patient GM-CSF administration was stopped at day 5 because of persistent high fever (40 ^{0}C). However, fever remained high after cessation of GM-CSF administration. The third patient showed rapidly progressive pulmonary infiltrates on the chest X-ray, which correlated with clinical pulmonary deterioration and a fast recovery of leukocytes in the peripheral blood (day 2: 0.08x10^{9}/l; day 5: 2.0x10^{9}/l) during the use of netilmycin, cefuroxime, vancomycin, co-trimoxazol, and amfotericin-B. Therefore, GM-CSF administration was discontinued after 5 days. High-dose prednisolone resulted in stabilization of the clinical situation. In the placebo group progressive jaundice occurred in one patient on day 3 but disappeared after discontinuation of clonazepam, ranitidine, and placebo. A tumor relapse within one month after the end of the study occurred in two patients (one SCLC, one non-Hodgkin's lymphoma) in the placebo group and none in the GM-CSF group.

Cytokine release during GM-CSF treatment

All of four tested patients in the GM-CSF group with gram-positive septicaemia showed increased IL-6 serum levels at the start of the study (table 4). In addition, one of four patients tested with gram-positive blood cultures (patient no.1, who died within five hours of GM-CSF administration), showed an increased TNF-α serum level. Intravenous GM-CSF administration did not affect TNF-α and IL-6 serum levels uniformly during the first hours of GM-CSF administration in these patients, although the IL-6 serum level did increase twofold in patient no.1 during this time period. The fifth patient (patient no.5) in this group with gram-positive septicaemia showed elevated TNF-α and IL-6 serum levels after one and four hours of intravenous GM-CSF administration. However, a sample at the start of GM-CSF administration, was not obtained. No signs of a possible threatening septic shock were observed in this patient. All five patients showed low monocyte counts (range 0-0.08x10^{9}/l).

Table 4. TNF-α and IL-6 serum levels in patients with gram-positive septicaemia treated with intravenous GM-CSF

	Patient					Hours since start of GM-CSF administration
	1	2	3	4	5	
TNF-α	39	6.9	<5	<5	ND	0
(ng/l)	53	8.7	<5	<5	75	1
	47	ND	37	<5	114	4
IL-6	1000	40	190	320	ND	0
(U/ml)	1500	45	110	370	180	1
	2100	ND	50	60	210	4

Blood cultures: Staphylococcus aureus (patient 1); Enterococcus faecalis (patient 2); Streptococcus mitis (patients 3, 4); hemolytic Streptococcus group C (patient 5).
Normal values: TNF-α < 5 ng/l, IL-6 < 20 U/ml. ND = not done.

DISCUSSION

Ten to twenty percent of patients treated with cytotoxic drugs will develop infectious complications during one of the chemotherapy courses (28). In view of this incidence it may be viewed as inefficient to treat all patients on chemotherapy with a hematopoietic growth factor to circumvent bone marrow hypoplasia. However, a CSF may be indicated in patients with an infection, especially since the severity of the infection is determined by the period of leukopenia (14). The present double-blind study in patients whose treatment with a standard dose of chemotherapy was complicated by fever and leukopenia demonstrates a significantly larger increase of the neutrophils during the first days of GM-CSF treatment than in the control group. In addition, it was shown that over the whole treatment period the increase in neutrophils for the GM-CSF group was significantly larger than in the control group, a fact suggesting that GM-CSF not only affected the mobilization of mature cells from the bone marrow, but also influenced the bone marrow restoration. In contrast, another pattern was noticed in the patients treated with GM-CSF within eight days of ablative chemotherapy and ABMT. No difference in the neutrophil recovery at days two and seven was observed in the GM-CSF versus the placebo group. The difference in responsiveness between the ABMT and

non-ABMT groups can be explained by a lower number of residual hematopoietic progenitor cells in the former group, in which the patients received more intensive chemotherapy. Nissen et al. observed comparable results in patients with aplastic anaemia treated with GM-CSF (29). The patients with the most severe degree of aplastic anaemia did not respond to GM-CSF.

The data of patients treated with ablative chemotherapy and ABMT suggest that a short course of GM-CSF may be of little benefit after ABMT. It is also consistent with the time now known to be required, even with GM-CSF treatment, of 10-12 days to achieve a leukocyte response after ABMT (8). GM-CSF administration may be worthwhile in patients treated with a standard-dose chemotherapy, since it enhanced the neutrophil recovery. This, in turn may shorten the period of antibiotic administration and reduce the morbidity and mortality.

Antman et al. have shown that 2.8 μg/kg per day GM-CSF administered by continuous intravenous infusion is an adequate dose for enhancing leukocyte recovery during chemotherapy-related myelosuppression (4). Peters et al. (30) have reported a significant reduction of neutrophil migration to a site of sterile inflammation in patients receiving GM-CSF by continuous infusion, which could be a disadvantage in GM-CSF-treated patients with severe infections. However, additional in vivo studies are required to determine the clinical significance of this observation since control of infections has proceeded effectively in patients during GM-CSF treatment. Furthermore, GM-CSF administration may be followed by an immediate transient fall in platelet count (31), which may imply an increased risk of bleeding. Therefore, early platelet transfusion is indicated in severe thrombocytopenic patients during the first hours of GM-CSF administration.

In contrast with data published by Nimer et al. (32), we did not observe a significant reduction in serum cholesterol levels during GM-CSF administration. This may be related to the relative low cholesterol levels in our patients at the start of the study compared with the cholesterol levels in patients with aplastic anemia studied by Nimer et al.

On theoretical grounds, the use of GM-CSF could have clinical disadvantages in patients with septicaemia, since in vitro data have shown an increased release of TNF-α in the presence of endotoxin or exotoxin and GM-CSF (26, 33). This was not seen in our patients. Two out of five patients with gram-positive septicaemia had increased TNF-α serum levels, which did not rise during the first hours of GM-CSF treatment. In addition, it was shown that the IL-6 levels, which were increased in most of the patients at the start of the study, did not rise during the first hours of GM-CSF administration. These in vivo data suggest that cytokine release will not further augment during gram-positive septicaemia and GM-CSF administration.

In summary, we conclude that a short period of GM-CSF administration may be of benefit in patients with fever and leukopenia since GM-CSF accelerates the neutrophil recovery. Longer periods of GM-CSF are required in patients treated with ablative chemotherapy and ABMT. An extended trial is needed to study the potential of GM-CSF to reduce morbidity and mortality resulting from infection, antibiotic administration or duration of hospitalization. We further conclude that patients with leukopenia and gram-positive septicaemia who are treated

with low-dose GM-CSF are not exposed to additional risks for septic shock related to cytokine release.

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Chapter 3

A DOUBLE-BLIND PLACEBO-CONTROLLED STUDY WITH GM-CSF DURING CHEMOTHERAPY FOR OVARIAN CARCINOMA

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SUMMARY

In a placebo-controlled double-blind dose-finding trial, fifteen patients with ovarian cancer stage III or IV received daily s.c. 1.5, 3, or 6 μg/kg recombinant human Granulocyte-Macrophage Colony-Stimulating Factor (rhGM-CSF). At each dose step three patients received rhGM-CSF, and two placebo. Chemotherapy comprised 6 cycles of carboplatin 300 mg/m^2 and cyclophosphamide 750 mg/m^2 i.v. bolus on day 1 every four weeks. GM-CSF, given on days 6-12 on an outpatient basis, raised the mean leukocyte count on days 7, 10, and 15 and the mean neutrophil count on days 7 and 10 at all dose levels as compared with the control group. Neutrophil counts of less than 0.5x10^9/l occurred in 20/22 cycles in the control group and in 5/17 cycles at the 6 μg/kg/day GM-CSF dose level ($p < 0.0005$). In comparison with the control group, the mean eosinophil count was higher on days 10 and 15 at all GM-CSF doses, as was the mean monocyte count on day 15. The mean platelet count was raised at the 3 and 6 μg GM-CSF doses on days 15 and 22. Chemotherapy dose reduction or postponement due to myelotoxicity occurred in 9/28 cycles in the placebo group versus 5/44 cycles in the GM-CSF group (NS). Local skin infiltrates at the GM-CSF injection sites occurred in 8/9 patients, leading to premature removal from the study of two patients. Capillary leakage of ^{131}I-albumin was increased in all patients five days after the first chemotherapy course, but was not significantly affected by four days of GM-CSF treatment. Tumor Necrosis Factor-alpha and C-reactive protein serum levels increased during GM-CSF administration at the 6 μg dose level, but Interleukin-6 serum levels were not affected.

We conclude that a dose of 3 and 6 μg/kg/day GM-CSF reduces the severity of neutropenia and thrombocytopenia after carboplatin-cyclophosphamide. This GM-CSF dose does not induce additional capillary leakage.

INTRODUCTION

Recently, several colony stimulating factors (CSFs) have been cloned (1-3) which created the opportunity to test in-vivo whether these CSFs can ameliorate chemotherapy-induced leukopenia or accelerate bone marrow restoration (4-8). One of these CFSs, Granulocyte-Macrophage CSF (GM-CSF), possesses stimulating activities for granulocyte-macrophage, eosinophilic and megakaryocytic progenitor cells in-vitro (9-13). In addition, GM-CSF can modify the functional activity of mature cells such as increased monocyte cytotoxicity through a Tumor Necrosis Factor-dependent mechanism (14).

In advanced ovarian cancer, platinum-containing compounds and alkylating agents are the most active drugs (15, 16). The second generation platinum-analogue carboplatin demonstrated definite activity in ovarian cancer (17, 18). In contrast to cisplatin, it is only minimally nephro-, oto-, and neurotoxic, but does have especially myelosuppression as a side effect. Carboplatin is therefore a logical drug to combine with CSFs. Since there seems to be a dose-response relation for carboplatin in ovarian cancer (19, 20), and the tumor response also seems related to the extent of bone marrow suppression (21), dose escalation plus marrow protection with GM-CSF should give better treatment results.

For a wider application of hematopoietic growth factors, it is essential to demonstrate their effect and safety when given to outpatients during consecutive cycles.

In the present double-blind placebo-controlled study in patients treated for ovarian cancer, we investigated the tolerability and efficacy of GM-CSF, administered s.c. to outpatients during repeated chemotherapy courses. In addition, the effect of GM-CSF on Tumor Necrosis Factor-alpha (TNF-α), Interleukin-6 (IL-6) and C-reactive protein (CRP) serum levels was measured, as GM-CSF can induce the in-vitro release of TNF-α from monocytes (14, 22) and possibly stimulates the in-vivo release of IL-6 (23). As GM-CSF, at higher doses, can induce capillary leakage (4, 6), the effect of GM-CSF on capillary leakage of ^{131}I albumin was studied by ^{131}I albumin disappearance.

The results demonstrate that at the dose levels of 3 and 6 μg/kg/day GM-CSF, the leukocyte, neutrophil, monocyte, eosinophil and platelet counts were increased compared with the control group. Furthermore, we present evidence that GM-CSF can augment TNF-α release in-vivo and that at doses up to 6 μg/kg/day GM-CSF does not induce additional capillary leakage.

MATERIALS AND METHODS

Fifteen consecutive patients, between 18 and 70 years of age, with Stage III-IV (staging according to FIGO) ovarian cancer and eligible for treatment with chemotherapy could be entered in the study. Patients with severe heart, lung, liver (serum total bilirubin $\geq$ 40 μmol/l),

or kidney impairment (creatinine clearance $\leq$ 60 ml/min) were excluded from the study, as were patients with a WHO performance score 3-4. Patients were not previously treated with chemotherapy or radiotherapy. All patients initially underwent tumor-reductive surgery before the start of chemotherapy.

Combination chemotherapy comprised 6 courses of carboplatin (300 mg/m^2) and cyclophosphamide (750 mg/m^2), both given on day 1 every four weeks on an outpatient basis. Carboplatin (Bristol-Myers, Troisdorf, West Germany) was dissolved in 250 ml 5% dextrose and infused over 30 min. Cyclophosphamide (ASTA Pharma A.G., Frankfurt, West Germany) was dissolved in 250 ml 0.9% saline and given i.v. over 15 min. Dose modification was only applied for nadir blood counts. Carboplatin dosage was reduced to 75% for nadir levels of leukocytes $< 1x10^9$/l, or platelets $< 50x10^9$/l. If this occurred again, cyclophosphamide was also reduced to 75% in the next cycle. Carboplatin dosage was reduced to 50% and cyclophosphamide dosage was reduced to 75% for nadir levels of leukocytes $< 0.5x10^9$/l, or platelets $< 25x10^9$/l. When leukocyte levels $> 3x10^9$/l and platelets $> 100x10^9$/l were reached, full chemotherapy doses were given. Treatment was postponed, up to a maximum of four weeks, for insufficient bone marrow recovery (leukocytes $< 3x10^9$/l or platelets $< 100x10^9$/l).

The study was a double-blind randomized placebo-controlled dose-finding trial, and the patients were divided into three groups of five patients each. The groups received 1.5, 3, or 6 μg/kg/day GM-CSF or placebo after each chemotherapy course. In each group, three patients received GM-CSF and two patients placebo. GM-CSF or placebo was administered once daily s.c. for seven days (days 6-12), starting five days after each chemotherapy course (day 1). This time interval was chosen as the serum platinum still present at that moment is mainly bound to protein and is unlikely to react with bone marrow stem cells (24). Patients were hospitalized only during the first GM-CSF or placebo course. GM-CSF or placebo dosages were not adjusted for side effects.

Recombinant human glycosylated mammalian derived GM-CSF, with a specific activity of $8x10^6$ U/mg protein, was provided by Sandoz/Schering Plough (Basle, Switzerland). The vials, containing 0.216 mg glycosylated GM-CSF, were reconstituted with 2 ml of the enclosed vehicle. Lyophilized placebo was also supplied in identical vials by Sandoz/Schering Plough. GM-CSF or placebo was injected s.c. once daily into the upper leg. If the injected volume exceeded 3 ml, the total volume was divided over two sites on the upper legs.

Relaparotomy was carried out in patients with a complete clinical remission after six chemotherapy cycles. If tumor progression occurred during the study, the patient was taken off the study. The study was approved by the Medical Ethical Committee of the University Hospital of Groningen. All patients gave their informed consent for the study.

Blood counts, including differential and reticulocyte counts, were performed on day 1, 7, 10, 15, and 22 during each cycle. Liver and renal functions were determined at day 1, 7, 15, and 22. Serum levels of cholesterol, sodium, potassium, calcium, total protein, and albumin

were determined at the same times. Blood pressure, temperature, body weight, and heart rate were measured on days 1 and 15. In addition, patients frequently measured their temperature at home.

Creatinine clearance was calculated from the creatinine levels in 24-hour urine and in serum. CA125 serum levels were measured before entry and on days 1 and 15 during each cycle by a commercially available enzyme-linked immunosorbent assay (Abbott, Chicago, U.S.A.).

The transcapillary escape rate of ^{131}I albumin (TER) was measured in 14 patients (five in the control group, nine in the GM-CSF group) five days after the first cycle of chemotherapy, before the start of GM-CSF, and after four days of GM-CSF. The methods were as described previously (25). The tracer, ^{131}I-labelled human serum albumin (^{131}I-HSA, IRE, Fleurs, Belgium), was injected into an arm vein. The injected dose of ^{131}I-HSA was approximately 200 kBq. Five-ml blood samples in heparinized blood collecting tubes were taken from the contralateral cubital vein 0, 5, 10, 15, 20, 30, 40, 50, 60, 75, 90, 105, and 120 min after injection of ^{131}I-HSA. The evening before the test, potassium iodine was administered orally to prevent ^{131}I uptake by the thyroid gland. The TER test was performed during steady state. Patients remained supine during the test. The TER (%/h) was calculated from the slope of the ^{131}I-albumin disappearance curve by a least squares fit of the data with a monoexponential function. Normal values for TER of ^{131}I-albumin are 3.8 $\pm$ 1.8 %/h (25).

TNF-α, IL-6 and CRP serum levels were measured in five patients during one cycle (control group one patient, 1.5 μg GM-CSF one patient, 6 μg GM-CSF three patients). The serum levels were studied on day 1 before chemotherapy, and on days 7, 10, 13, and 15. The samples on days 7, 10 and 13, were taken 22 h after the last GM-CSF or placebo injection. TNF-α (normal value $<$ 5 ng/l) was measured with a radio-immunoassay (Medgenix, Brussels, Belgium), IL-6 (normal value $<$ 20 U/ml) with the B9 bioassay (26), and CRP (normal value $<$ 2 mg/l) with an enzyme-linked immunosorbent assay (27).

Statistical analysis

The Student's t-test, the chi-square test for small numbers, the Wilcoxon rank sum test, and the log rank test were used for statistical analysis. Only p values $<$ 0.05 were considered significant. Unless otherwise stated, the Student's t-test was used for statistical analysis.

RESULTS

Patient characteristics

In the GM-CSF group nine patients presented with stage III ovarian cancer, while in the control group four patients were diagnosed with stage III and two patients with stage IV ovarian cancer. The median age of patients in the GM-CSF and control group was 57 years (range 31-

66) and 58 years (range 43-64), respectively. Creatinine clearance at entry was 81±21 ml/min, (x±SD) in the control group and 84±6 ml/min, 98±44 ml/min and 91±26 ml/min, respectively in the 1.5, 3 and 6 μg/kg/day GM-CSF dose groups (NS).

Hematological recovery

Hematological recovery was determined for all fully dosed chemotherapy cycles (six reduced cycles in the control group and two in the GM-CSF group were evaluated for GM-CSF toxicity only). GM-CSF significantly raised the mean leukocyte count (Table 1) on days 7, 10, and 15 and the mean neutrophil count (Table 2) on days 7 and 10 at all three GM-CSF dose levels, as compared with the control group. On day 22, the leukocyte and neutrophil count at 6 μg/kg/day were increased in comparison with the control group. The neutrophil count on day 15 was not significantly raised at any of the GM-CSF dose levels. However, a neutrophil count less than 0.5×10^9/l occurred in 20 out of 22 cycles in the control group and in 7 out of 10, 8 out of 13, and 5 out of 17 cycles at the 1.5, 3 and 6 μg/kg/day GM-CSF dose levels, respectively ($p < 0.0005$ for the 6 μg dose level compared to the control group, chi-square test). Seven days of GM-CSF did not affect the leukocyte and neutrophil count on day 29 (the day the next chemotherapy course was planned). The effect of GM-CSF on the leukocyte count remained notable through consecutive chemotherapy courses. The mean area under the curve for percentage decrease in leukocyte counts during course one was: control group 1516±239 % decrease.days (x±SD, n=6), and 878±330, 502±504, and 436±42 % decrease.days (n=3) for the 1.5, 3, and 6 μg/kg/day GM-CSF dose levels, respectively ($p < 0.001$ for cycle one of the GM-CSF group compared to cycle one of the placebo group, Wilcoxon rank sum test). Mean area under the curve for percentage decrease in leukocyte counts during course six in the GM-CSF group was 464±390 (n=6), % decrease.days with $p < 0.005$ compared to cycle one of the control group (Wilcoxon rank sum test).

A striking effect of GM-CSF on the eosinophil counts was seen (Table 3). The absolute eosinophil count was higher at both day 10 and day 15 at all three GM-CSF dose levels compared to the control group. After cessation of GM-CSF treatment the increase persisted till day 22 in the 1.5 and 6 μg/kg/day GM-CSF group. The absolute monocyte counts on day 15 were raised at all three GM-CSF dose levels compared to the control group (Table 4). Furthermore, on days 7 and 10, the monocyte count at the 6 μg/kg/day GM-CSF dose step was increased compared to the control group. On days 7, 10, and 15 the monocyte count at the 6 μg dose level was increased in comparison with the 1.5 μg dose level, as was the monocyte count at the 6 μg dose level on day 10 compared to the 3 μg dose level. The lymphocyte count was only raised on day 15 at the 3 and 6 μg/kg/day GM-CSF dose steps in comparison with the control group (control group $0.9 \pm 0.4 \times 10^9$/l, n=16; 3 μg GM-CSF $1.27 \pm 0.28 \times 10^9$/l, n=11; 6 μg GM-CSF $1.19 \pm 0.47 \times 10^9$/l, n=13). Basophil and reticulocyte counts were not affected by GM-CSF.

Table 1. Mean leukocyte count during fully dosed chemotherapy cycles

	Leukocytes ($x\ 10^9/l$)							
	Controls		GM-CSF dose ($\mu g/kg/day$)					
			1.5		3		6	
Day	Mean±SD	n[a]	Mean±SD	n	Mean±SD	n	Mean±SD	n
1	4.4±1.5	19	5.2±2.8	8	5.0±1.4	11	4.4±0.7	14
7	3.0±0.7	21	4.6±1.8[f]	11	5.6±1.8[f]	13	4.8±2.4[d]	16
10	1.9±0.6	22	2.9±1.7[c]	9	3.6±1.5[f]	13	4.7±2.5[f]	16
15	1.6±0.9	22	2.7±0.6[d]	10	2.8±1.5[d]	13	3.1±1.0[f]	17
22	3.0±1.1	21	3.9±1.8	9	3.3±1.0	12	3.6±0.9[b]	16

[a]: Number of evaluable courses, [b]: $p < 0.05$ compared to control group, [c]: $p < 0.02$ compared to control group, [d]: $p < 0.005$ compared to control group, [f]: $p < 0.001$ compared to control group.

Table 2. Mean neutrophil count during fully dosed chemotherapy cycles

	Neutrophils ($x\ 10^9/l$)							
	Controls		GM-CSF dose ($\mu g/kg/day$)					
			1.5		3		6	
Day	Mean±SD	n[a]	Mean±SD	n	Mean±SD	n	Mean±SD	n
1	3.15±1.52	14	3.38±2.78	8	3.78±1.34	10	2.77±0.70	13
7	1.99±0.56	20	3.37±1.38[d]	10	4.49±1.83[d]	12	3.36±1.99[c]	14
10	0.95±0.53	18	2.07±1.37[c]	6	2.18±1.17[d]	13	3.50±2.11[d]	14
15	0.49±0.61	16	0.64±0.74	8	0.70±0.92	11	0.71±0.47	16
22	1.37±0.65	16	2.20±1.59	7	1.74±0.70	11	1.80±0.44[b]	15

[a]: Number of evaluable courses, [b]: $p < 0.02$ compared to control group, [c]: $p < 0.01$ compared to control group, [d]: $p < 0.001$ compared to control group.

Table 3. Mean eosinophil count during fully dosed chemotherapy cycles

	Eosinophils ($x\ 10^9$/l)							
	Controls		GM-CSF dose (μg/kg/day)					
			1.5		3		6	
Day	Mean±SD	n[a]	Mean±SD	n	Mean±SD	n	Mean±SD	n
1	0.10±0.16	14	0.10±0.09	8	0.04±0.05	10	0.09±0.09	13
7	0.12±0.09	20	0.16±0.11	10	0.08±0.08	12	0.18±0.12	14
10	0.06±0.06	18	0.44±0.15[d]	6	0.29±0.22[d]	13	0.30±0.22[d]	14
15	0.02±0.03	16	0.51±0.24[d]	8	0.54±0.33[d]	11	0.65±0.59[d]	16
22	0.05±0.03	16	0.11±0.10[b]	7	0.07±0.04	11	0.18±0.17[c]	15

[a]: Number of evaluable courses, [b]: $p < 0.05$ compared to control group, [c]: $p < 0.01$ compared to control group, [d]: $p < 0.001$ compared to control group.

Table 4. Mean monocyte count during fully dosed chemotherapy cycles

	Monocytes ($x\ 10^9$/l)							
	Controls		GM-CSF dose (μg/kg/day)					
			1.5		3		6	
Day	Mean±SD	n[a]	Mean±SD	n	Mean±SD	n	Mean±SD	n
1	0.24±0.17	14	0.37±0.39	8	0.26±0.13	10	0.28±0.07	13
7	0.05±0.04	20	0.02±0.03	10	0.11±0.13	12	0.09±0.05[b]	14
10	0.03±0.03	18	0.07±0.08	6	0.09±0.14	13	0.19±0.09[c]	14
15	0.19±0.10	16	0.34±0.15[b]	8	0.47±0.26[c]	11	0.59±0.20[c]	16
22	0.19±0.10	16	0.14±0.08	7	0.25±0.08	11	0.20±0.07	15

[a]: Number of evaluable courses, [b]: $p < 0.02$ compared to control group, [c]: $p < 0.001$ compared to control group.

The mean platelet count on days 15 and 22 was significantly higher at the 3 and 6 μg/kg/day GM-CSF dose level compared to the control group (Table 5).

The chemotherapy dose was reduced in 6/28 cycles in the placebo group versus 2/44 (0/11, 1/15, and 1/18 cycles at the 1.5, 3, and 6 μg/kg day dose levels respectively) in the GM-CSF group (Table 6). Chemotherapy delay due to insufficient bone marrow recovery (leukocyte count < 3.0 x10^9/l) was necessary in 3/28 cycles in the placebo group. This resulted in premature removal of two patients from the study. In the 1.5 μg/kg/day GM-CSF group, postponement was indicated after cycles 3 and 5 (2 weeks each) for one patient. In the 6 μg/kg/day GM-CSF group treatment delay was necessary in one patient after cycle 4 (2 weeks). In addition, one patient in the 1.5 μg/kg/day GM-CSF dose group was taken off the study after two cycles of chemotherapy because of tumor progression. Two out of six patients in the control group (33%) and four out of nine patients in the GM-CSF group (45%) completed the six cycles without chemotherapy dose reduction and postponement (NS). If chemotherapy delay, chemotherapy dose reduction, and premature discontinuation of GM-CSF administration were scored as events and tumor progression was scored as lost to follow-up, in the log rank test the difference was not significant between the control and the GM-CSF group.

Table 5. Mean platelet count during fully dosed chemotherapy cycles

	Platelets (x 10^9/l)							
	Controls		GM-CSF dose (μg/kg/day)					
			1.5		3		6	
Day	Mean±SD	n[a]	Mean±SD	n	Mean±SD	n	Mean±SD	n
1	293±81	19	221±69	8	242±87	11	314±105	14
7	242±53	18	165±42	9	186±53	12	213±105	14
10	155±42	21	129±28	8	139±52	12	208±109	14
15	61±43	22	56±17	9	102±36[c,f]	13	125±114[b]	17
22	167±98	19	138±96	8	254±111[b,d]	12	259±162[b]	15

[a]: Number of evaluable courses, [b]: $p < 0.05$ compared to control group, [c]: $p < 0.01$ compared to control group, [d]: $p < 0.05$ at 3 μg/kg/day GM-CSF compared to 1.5 μg/kg/day GM-CSF, [f]: $p < 0.005$ at 3 μg/kg/day GM-CSF compared to 1.5 μg/kg/day GM-CSF.

Table 6. Reasons for discontinuation of study, chemotherapy dose reduction and chemotherapy postponement

	GM-CSF			Control
	1.5	3	6 μg/kg/day	
Premature discontinuation of study (patients)				
- Tumor progression	1	-	-	-
- Prolonged myelotoxicity	-	-	-	2
- Infiltrate injection site	1	1	-	-
Number of evaluable courses	11	15	18	28
Dose reduction (courses)				
- Carboplatin 25% reduction	-	1	-	6
- Cyclophosphamide 25% + carboplatin 50% reduction	-	-	1	-
Chemotherapy postponement				
- Number of patients	1		1	2
- Number of courses with				
1 week postponement	-	-	-	1
2 weeks postponement	2	-	1	2
Postponement resulting in removal from the study	-	-	-	2

Four patients in the GM-CSF group, at relaparotomy, had a partial remission (placebo group three patients) and two patients had a complete remission (placebo group one patient) (NS). CA125 serum levels normalized after six chemotherapy courses in five out of six patients in the GM-CSF group and in four of the six controls who completed treatment (NS).

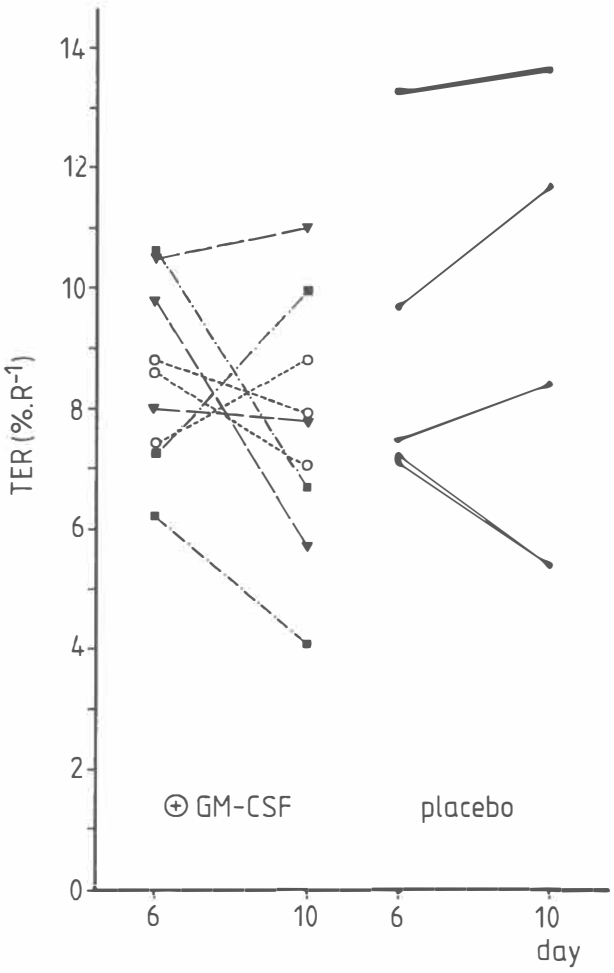

Figure 1. Transcapillary escape rate (TER) of ^{131}I albumin in patients treated with chemotherapy and GM-CSF. TER day 6: control group 9.0±2.6 %/h (n=5); 1.5 μg/kg/day GM-CSF 8.3±0.8 %/h (n=3); 3 μg/kg/day GM-CSF 9.4±1.3 %/h (n=3); 6 μg/kg/day GM-CSF 8.0±2.3 %/h (n=3, x±SD), NS. TER day 10: control group 8.9±3.7 %/h; 1.5 μg/kg/day GM-CSF 7.9±0.9 %/h; 3 μg/kg/day GM-CSF 8.2±2.7 %/h; 6 μg/kg/day GM-CSF 6.9±2.9 %/h (NS).
○ = 1.5 μg/kg/day GM-CSF, ▾ = 3 μg/kg/day GM-CSF, ■ = 6 μg/kg/day GM-CSF.
NS = not significant.

Side effects

A local reaction at the GM-CSF injection site was noticed in all patients during one of the GM-CSF courses. Local redness was observed in all patients. Two patients (3 and 6 μg GM-CSF dose step) experienced a rash on the chest and back and generalized itching during the first and second GM-CSF course respectively. Eight patients developed local skin infiltrates at the injection site during one or two GM-CSF courses. Biopsies of the infiltrates from two patients showed perivascular lymphocytic infiltrates with relatively large numbers of eosinophils. GM-CSF administration (1.5 and 3 μg/kg/day) was discontinued in two patients because of the severity of the local infiltrates during the third GM-CSF course. In the other patients the local reaction became less severe or disappeared during the following GM-CSF courses. There was no relationship between the severity of the local reaction and the GM-CSF dose or eosinophil counts. No fever or hypotension was observed in any of the patients. The only infection observed was one of the lower urinary tract in the placebo group.

Cholesterol levels at day 15 in patients treated with GM-CSF were lower than in the control group (GM-CSF group: 4.91 ± 0.43 mmol/l, $x \pm SD$, $n = 19$; control group: 5.35 ± 0.95, $n = 10$; $p < 0.05$). Cholesterol levels were not significantly different on day 1 (GM-CSF group 6.27 ± 0.59 mmol/l; control group 5.9 ± 0.97 mmol/l), and day 7 (GM-CSF group 5.12 ± 0.54 mmol/l; control group 4.99 ± 1.15 mmol/l) . GM-CSF did not affect kidney and liver function.

Capillary leakage of serum albumin was increased before the start of GM-CSF infusion in all patients tested five days after the first chemotherapy course (Fig. 1). Capillary leakage on day 10 at the three GM-CSF dose steps was not higher than in the placebo group, suggesting that at these dosages, GM-CSF did not further increase capillary leakage of serum albumin, although there is a considerable patient to patient variation.

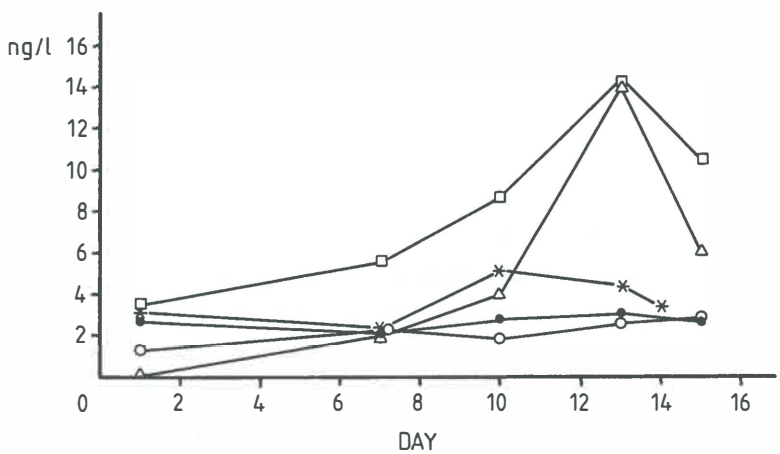

Figure 2. TNF-α serum levels during GM-CSF administration in five individual patients. ● = control group, ○ = 1.5 μg/kg/day GM-CSF, ▵,□,★ = 6 μg/kg/day GM-CSF.

Cytokine release

TNF-α and CRP serum levels in the five patients tested were normal before chemotherapy administration (day 1). Levels did not increase during the administration of placebo or 1.5 μg/kg/day GM-CSF in the two patients tested (Figure 2 and 3). However, at the 6 μg/kg/day GM-CSF dose step TNF-α and CRP serum levels did increase during GM-CSF administration in the three patients tested. The levels decreased after cessation of GM-CSF. These effects could not be ascribed to IL-6 release since no significant difference in IL-6 levels in patients at the 6 μg GM-CSF dose level were observed on day 5 and day 13 ($<$ 12 U/ml at both days). No signs of infection or local infiltrates at the injection site were noticed during the period of measurement in these patients.

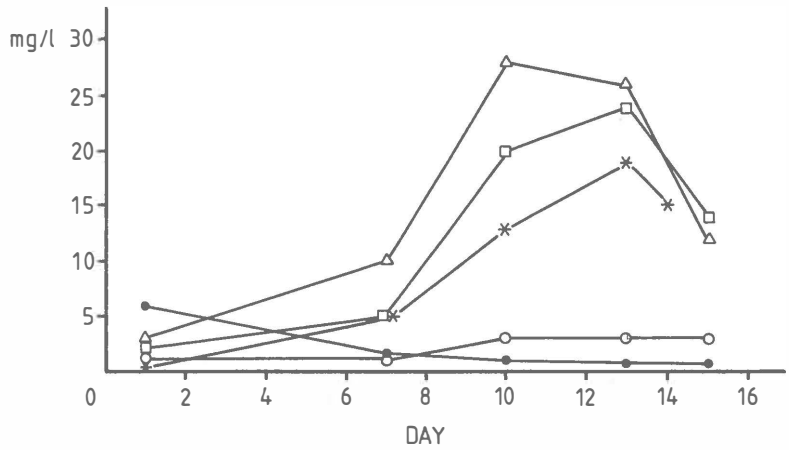

Figure 3. CRP serum levels during GM-CSF administration.
● = control group, ○ = 1.5 μg/kg/day GM-CSF, ▵,□,★ = 6 μg/kg/day GM-CSF.

DISCUSSION

The study reported here is the first double-blind placebo-controlled trial with increasing doses of GM-CSF administered s.c. on an outpatient basis during consecutive cycles in previously untreated patients with ovarian carcinoma. Several studies with GM-CSF after ablative chemotherapy and autologous bone marrow transplantation (ABMT) are reported (6, 7, 28). Some studies are described after chemotherapy without ABMT (4, 29, 30). Antman et al. studied patients with sarcomas, with escalating doses of GM-CSF administered intravenously before and after a first cycle of chemotherapy (4). A preliminary report of Edmonson et al. describes data after s.c. GM-CSF therapy (29). Gianni et al. studied the effect of continuously infused GM-CSF in patients with breast cancer or non-Hodgkin's lymphoma after high-dose

cyclophosphamide (30). In the present study, the leukocyte and neutrophil counts were less markedly depressed than in the control group at all three GM-CSF dose levels (Tables 1 and 2). The effect of GM-CSF on the leukocyte count, as expressed by the area under the curve for percentage decrease in leukocyte counts during each cycle, was still present in the sixth cycle. The elevation of neutrophils is important, as severe infections in neutropenic patients occur especially at a neutrophil count below $0.5x10^9$/l (31). A neutrophil count of less than $0.5x10^9$/l occurred significantly less frequently at the highest GM-CSF dose level.

Apart from a raised neutrophil count, raised eosinophils also contributed extensively to the leukocyte count in this study (Table 3). Aglietta et al. (32) observed little effect on eosinophils after three days continuous i.v. infusion of GM-CSF (8 μg/kg/day) in patients with solid tumors or non-Hodgkin's lymphoma. Herrmann et al. (33) noticed at 250-1000 $\mu g/m^2$/day GM-CSF by continuous i.v. infusion a four- to five-fold increase in eosinophils after five days of GM-CSF administration in patients not treated with chemotherapy. Comparable results were obtained by Lieschke et al. (34) at a dose of 20 μg/kg/day GM-CSF, administered s.c.. In contrast, a rise of eosinophils has not been described after high-dose chemotherapy and autologous bone marrow transplantation (6, 7). A rise in the lymphocyte count also was observed by Lieschke et al. (34) at several GM-CSF dose levels and by Phillips et al. (35) at 100 and 500 $\mu g/m^2$/day GM-CSF.

Different effects of GM-CSF on platelet levels have been reported. In mice, platelet recovery after irradiation was accelerated by GM-CSF (36). Gianni et al. reported that patients treated with 14 days of continuous GM-CSF infusion (5.5 μg/kg/day) after high-dose cyclophosphamide required less prophylactic platelet transfusions (30). Of four studies performed with GM-CSF without chemotherapy, two studies showed no consistent rise in platelets (34, 35) and one study reported a decline in platelet levels during the first three days continuous GM-CSF infusion (4). In an additional study, a slight decrease during the first five treatment days and a significant increase during the following five days of GM-CSF treatment was observed (37). This positive effect on platelet levels did not occur in a second treatment cycle with GM-CSF, again without chemotherapy. No effect on platelets was observed when GM-CSF was instituted after high-dose chemotherapy and autologous bone marrow transplantation in two studies (6, 28). Nemunaitis et al. (7), however, observed earlier independence of platelet transfusions in some patients. Treatment with GM-CSF after chemotherapy in sarcoma patients showed a higher platelet nadir than in the control group (4). Finally, Edmonson et al. (29) noted an amelioration of carboplatin-cyclophosphamide induced thrombocytopenia by GM-CSF. One should be cautious in interpreting platelet nadir results after carboplatin therapy without taking renal function into account. Platelet nadir after carboplatin treatment is closely related to the renal function (38, 39). In our study, with no significant difference in renal function between the different patient groups, the mean platelet count on day 15 was significantly higher at the two highest GM-CSF dose steps than in the placebo group (Table 5).

Although GM-CSF can stimulate some non-hematopoietic tumor cells (40), no evidence is available yet that this is the case for ovarian carcinoma cells (41). The tumor response rate, as determined by relaparotomy, was normal in the GM-CSF group, but the number of patients in our study is too small to enable final conclusions to be drawn.

The fact that GM-CSF increases the number and functional activity of monocytes may be favorable for tumor response. Activated monocytes can have a cytotoxic effect on ovarian cancer cells (42). This effect may be increased by GM-CSF, as GM-CSF increases in-vitro monocyte cytolytic activity and induces TNF secretion (14, 22, 43-45). The present study shows that TNF-α serum levels are increased during the administration of 6 μg/kg/day GM-CSF in three patients (Figure 2), presenting evidence that, in-vivo, TNF-α release is augmented by a relatively low dose GM-CSF. This effect may be dose-dependent. It was not related to any side effects, as side effects were not observed during the courses TNF-α, IL-6, and CRP serum levels were measured. The increase in CRP serum levels (Figure 3) supports the functional relevance of the TNF-α levels, since TNF-α release is accompanied by an acute-phase response.

Local reaction, ranging from redness to infiltrates leading to cessation of GM-CSF administration, was observed in all patients in the GM-CSF group. The local reactions disappeared, however, in most patients during the following courses with GM-CSF. In addition, a temporary generalized rash and pruritus was noticed in two patients. Lieschke et al. (34) observed local reaction in 43% of their patients after s.c. GM-CSF administration.

With ^{131}I albumin we showed that, even after only one dose of cyclophosphamide and carboplatin, the transcapillary leakage of albumin, as described in other studies of patients treated with chemotherapy (25, 46), is increased (Figure 1), but that no significant further increase occurred during treatment with GM-CSF. Although there was a considerable patient to patient variation, patients treated at the doses reported in this study therefore do not seem to be at additional risk for a capillary leakage syndrome during GM-CSF treatment. This is in contrast with earlier GM-CSF studies (4, 6), using high doses of GM-CSF (32-64 μg/kg).

As reported earlier, GM-CSF can affect serum cholesterol levels (47). In our study, no effect on serum cholesterol levels was measured after two days of GM-CSF administration. Three days after cessation of GM-CSF treatment cholesterol levels were lower in the GM-CSF group, but this effect disappeared before the following chemotherapy course.

The fact that fewer dose reductions and chemotherapy postponements were necessary in the GM-CSF group may result in a higher drug dose-intensity, but positive effects on tumor response percentage will have to be studied in a randomized study with a larger number of patients.

In conclusion, this study demonstrated that leukocyte, neutrophil, monocyte, eosinophil, and platelet counts increased at the 3 and 6 μg/kg/day GM-CSF dose levels, compared to a control group. The use of GM-CSF at these doses seems beneficial for patients with ovarian cancer treated with carboplatin and cyclophosphamide, as it may enable dose-intensification of

the chemotherapeutic drugs. This is important as tumor response on carboplatin and cyclophosphamide may be dose-dependent (21). In view of these findings, it may be worthwhile to examine a combination of GM-CSF and IL-3 in preventing myelotoxicity, as these CSFs act synergistically in stimulating hematopoiesis in primates (48). In addition we presented evidence that GM-CSF can augment TNF-α release, an effect that, in combination with a positive effect on the monocyte count, could augment tumor response. A larger randomized study, however, is needed to evaluate this effect.

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Chapter 4

EFFECTS OF INTERLEUKIN-3 AFTER CHEMOTHERAPY FOR ADVANCED OVARIAN CANCER

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SUMMARY

To define the maximum tolerated dose and to study whether recombinant human Interleukin-3 (rhIL-3) reduced chemotherapy-induced neutropenia and thrombocytopenia, twenty chemotherapy-naive patients with advanced ovarian cancer eligible for treatment with 6 cycles carboplatin-cyclophosphamide every four weeks (day 1) were entered in a phase I/II open, single centre trial. Cohorts of five patients received during seven days 1, 5, 10 or 15 μg/kg/day rhIL-3 (days 5-11) in cycles 1, 3 and 5 by continuous intravenous infusion or once daily subcutaneous administration. In control cycles 2, 4 and 6 no rhIL-3 was administered.

RhIL-3 significantly increased the recovery of leukocyte, neutrophil and platelet counts, especially at 5, 10 and 15 μg/kg rhIL-3. RhIL-3 also increased basophil, eosinophil, monocyte and lymphocyte counts at these dose steps. Effects on reticulocytes were limited. No difference in efficacy between sc and iv rhIL-3 treatment was found. Chemotherapy postponement for insufficient bone marrow recovery was necessary in 22/45 control cycles versus 2/49 rhIL-3 cycles ($p < 0.001$). Platelet transfusions were required in 7/45 control cycles versus 3/50 rhIL-3 cycles ($p < 0.5$). RhIL-3 up to doses of 10 μg/kg/day could be administered without severe side effects. At 15 μg/kg/day rhIL-3 headache was dose-limiting. Other side effects were fever, flu-like symptoms, nausea, skin rash, flushing, facial erythema and urticaria. Liver toxicity occurred in rhIL-3 and control cycles. RhIL-3 slightly raised tumor necrosis factor alpha, C-reactive protein and serum amyloid A plasma levels, while no effect on Interleukin-6 plasma levels was observed. RhIL-3 administered sc appears to be an interesting hematopoietic growth factor for reduction of chemotherapy-induced myelotoxicity.

INTRODUCTION

With the cloning of several hematopoietic colony stimulating factors (CSF's), the opportunity arose to explore whether these CSF's ameliorate chemotherapy-induced myelosuppresssion or accelerate bone marrow restoration (1-4). Granulocyte CSF (G-CSF) can reduce chemotherapy-related neutropenia, but has no effect on platelet recovery (5, 6). For Granulocyte-Macrophage CSF (GM-CSF) a reduction in chemotherapy-related neutropenia has been demonstrated (7-9). However, data concerning effects on platelets are inconsistent. Some studies demonstrated a positive effect of GM-CSF on the platelet recovery, whereas in other studies no effect was shown (7-10).

Interleukin-3 (IL-3) is a glycoprotein that promotes the survival, proliferation and differentiation of multipotential hematopoietic stem cells and of the committed progenitor cells of the megakaryocyte, granulocyte/macrophage, erythroid, eosinophil, basophil and mast cell lineages in vitro (11-14). It also affects mature cells by increasing cytotoxic functions or inducing release of cytokines, but does not affect the function of mature neutrophils (15, 16). In comparison with other CSF's, IL-3 has demonstrated to be a more potent stimulator of megakaryopoiesis in vitro (17, 18).

Recent clinical studies with recombinant human (rh) IL-3 administered to patients with advanced malignancies, myelodysplastic syndromes, aplastic anemia or bone marrow failure (19-22) demonstrated its multilineage stimulation of hematopoiesis in vivo. These effects were especially prominent in patients with normal bone marrow function (19). Therefore, rhIL-3 could be a potent CSF for the reduction of chemotherapy-induced neutropenia and thrombocytopenia.

In advanced ovarian cancer, platinum-derived compounds and alkylating agents are the most active drugs (23). Until recently cisplatin has been the mainstay in treatment of advanced ovarian cancer, however at the cost of side effects, such as nephro- and neurotoxicity (23). Carboplatin has demonstrated dose-related activity in this tumor (23, 24), but does have especially myelosuppression as a dose-limiting side effect. In a previous study we have demonstrated that GM-CSF reduced myelosuppression after carboplatin and cyclophosphamide for advanced stages of primary ovarian cancer (8).

In the present study in patients with ovarian cancer treated with the same chemotherapy, the tolerability and efficacy of rhIL-3, as well as the effect of rhIL-3 on cytokine release, was evaluated.

MATERIALS AND METHODS

Patients

Twenty consecutive patients, between 18 and 70 years of age, with Stage III-IV ovarian cancer

according to the International Federation of Gynecologists and Obstetricians (FIGO) and eligible for treatment with chemotherapy were entered. At entry a leukocyte count $\geq 3.0 \times 10^9$/l and a platelet count $\geq 100 \times 10^9$/l was required. Patients with severe heart, lung, liver (serum total bilirubin ≥ 40 μmol/l), or kidney impairment (creatinine clearance ≤ 50 ml/min) were excluded from the study, as were patients with a WHO performance score grade 3-4. Those previously treated with chemotherapy or on treatment with steroids, morphine, or cimetidine and analogues were not eligible for the study. Whenever possible, patients underwent tumor-reductive surgery before the start of chemotherapy.

Study design

Combination chemotherapy comprised six courses of carboplatin (300 mg/m^2) and cyclophosphamide (750 mg/m^2), both given iv on day 1 every four weeks on an outpatient basis. Carboplatin (Bristol-Myers Squibb, Princetown, USA), dissolved in 250 ml 5% dextrose, was infused over 30 min, cyclophosphamide (ASTA Pharma A.G., Frankfurt, Germany), dissolved in 250 ml 0.9% saline, over 15 min. Chemotherapy dose reduction was not applied. Treatment was postponed, up to a maximum of four weeks, when insufficient bone marrow recovery (leukocytes $< 3 \times 10^9$/l or platelets $< 100 \times 10^9$/l) occurred. Platelet transfusions were administered at a platelet count $< 20 \times 10^9$/l or when signs of bleeding tendency occurred.

Escherichia Coli derived nonglycosylated rhIL-3 ($2\text{-}10 \times 10^6$ U/mg protein) was provided by Sandoz (Basle, Switzerland) in vials of 150 μg (for iv) and 300 μg (for sc). For the daily iv infusion polypropylene syringes were used for each of two 12 hour infusions, containing half of the daily rhIL-3 dosage, 96 mg (2 mg/ml) human serum albumin and 0.9% saline with a total volume of 48 ml. RhIL-3 in the syringes remained stable for 24 h. For sc administration rhIL-3 was reconstituted with 1 ml sterile water. After instructions by the oncology nurse, rhIL-3 was injected sc in the upper leg, on an outpatient basis. If the volume exceeded 4 ml the dosage was devided over both upper legs.

In this phase I/II study the patients were divided into four groups of five patients each. After the first, third and fifth chemotherapy course, each group received 1, 5, 10 or 15 μg/kg/day rhIL-3 for seven days (day 5-11), starting four days after chemotherapy (day 1). To study a possible difference in tolerability the patients were randomized for either continuous iv or once daily sc rhIl-3 administration in cycle 1. In cycle 3 the route of administration was reversed. During cycle 5 all patients received rhIL-3 sc. For evaluation of hematological effects no rhIL-3 was administered in cycles 2, 4 and 6 (control cycles). The maximum tolerated dose (MTD) was defined as the dose level preceding that at which at least two patients experienced WHO Grade III or IV toxicity or life debilitating toxicity leading to discontinuation of rhIL-3 administration. Acetaminophen (max. 3 g/day) was given when headache, fever > 38.0 °C (measured axillary), or flu-like symptoms occurred.

If tumor progression occurred, the patient was taken off study. The study was approved by the Medical Ethical Committee of the University Hospital of Groningen. Informed consent

was obtained from all patients.

Blood counts, including differential counts, were performed on days 1, 8, 13, 15 and 22 during each cycle. Liver and renal functions and serum levels of sodium, potassium, calcium, total protein, and albumin were determined on days 1, 8, 15 and 22. Blood pressure, temperature, body weight and heart rate were measured daily during rhIL-3 administration and every two weeks during the control cycles. Creatinine clearance was calculated from the creatinine levels in 24-hour urine and in serum. Tumor necrosis factor alpha (TNF-α), Interleukin-6 (IL-6), C-reactive protein (CRP), and serum amyloid A (SAA) plasma levels were determined during iv and sc rhIL-3 administration in all patients. The plasma samples were taken on day 1 before chemotherapy, on day 5 (before rhIL-3 administration), and on days 6, 7, 11, and 14 or 15. TNF-α (detection limit 5 ng/l) was measured with a radio-immunoassay (Medgenix, Brussels, Belgium), IL-6 (normal value $<$ 10 U/ml) with the B9 bioassay (25), CRP (normal value $<$ 2 mg/l) and SAA (normal value $<$ 3 mg/l) with enzyme-linked immunosorbent assays (26). Urinary methylhistamine (normal value 0-150 μmol/mol creatinine) and methylimidazoleacetic acid (normal value 0.5-2.5 mmol/mol creatinine) levels in 24-hour urine were measured on days 1, 5 and 11 as described before (27).

Statistical analysis

The two tailed Student's t-test, the chi-square test for small numbers, the Kruskal-Wallis analysis of variance, and the Wilcoxon rank sum test were used for statistical analysis. P values $<$ 0.05 were considered significant. Unless otherwise stated, the two tailed Student's t-test was used for statistical analysis.

RESULTS

Patient characteristics

The median age of the patients entered in the study was 59.5 years (range 21-69). Sixteen patients presented with stage III ovarian cancer, while four patients were diagnosed with stage IV. Creatinine clearance (mean$\pm$SD) at entry was 100$\pm$14 ml/min, 89$\pm$28 ml/min, 117$\pm$24 ml/min, and 109$\pm$10 ml/min in the 1, 5, 10, and 15 μg/kg/day IL-3 dose groups, respectively (NS for all dose steps).

Hematological recovery

Fifty cycles with rhIL-3 (1 μg: 12 cycles; 5 μg: 13 cycles; 10 μg: 15 cycles; 15 μg: 10 cycles) and 45 control cycles were evaluable for hematological effects. Two cycles with rhIL-3 at 15 μg were not evaluable because of premature discontinuation of rhIL-3 due to toxicity. Progressive myelotoxicity in consecutive chemotherapy courses could not be demonstrated in this study, as the area under the curve for the neutrophils and platelets, expressed as percentage

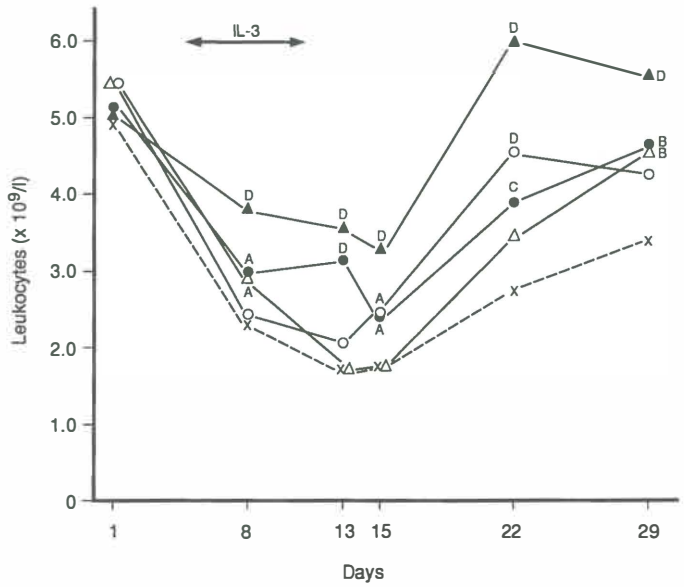

Figure 1. Mean leukocyte counts during rhIL-3 administration after chemotherapy.
P values compared to control cycles: a: $p<0.05$, b: $p<0.02$, c: $p<0.01$, d: $p<0.001$.
x = control cycles; ▵ = 1 μg rhIL-3; ○ = 5 μg rhIL-3; ▲ = 10 μg rhIL-3; ● = 15 μg rhIL-3.

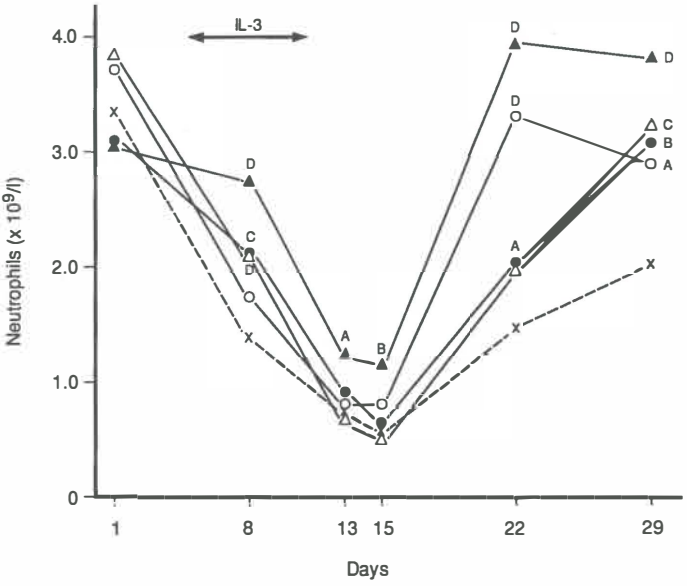

Figure 2. Mean neutrophil counts during rhIL-3 administration after chemotherapy.
P values compared to control cycles: a: $p<0.05$, b: $p<0.02$, c: $p<0.01$, d: $p<0.002$, e: $p<0.001$.
x = control cycles; ▵ = 1 μg rhIL-3; ○ = 5 μg rhIL-3; ▲ = 10 μg rhIL-3; ● = 15 μg rhIL-3.

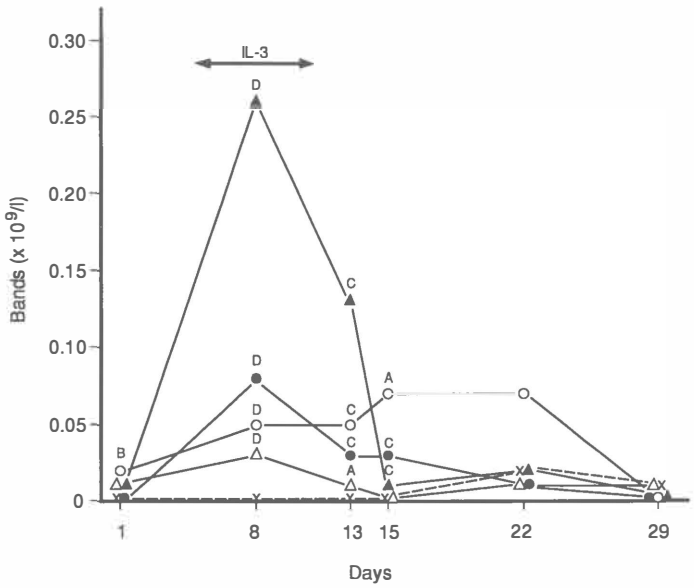

Figure 3. Mean banded neutrophil counts during rhIL-3 administration after chemotherapy.
P values compared to control cycles: a: $p<0.05$, b: $p<0.01$, c: $p<0.002$, d: $p<0.001$.
x = control cycles; ▵ = 1 μg rhIL-3; ○ = 5 μg rhIL-3; ▲ = 10 μg rhIL-3; ● = 15 μg rhIL-3.

change from day 1, did not differ between control cycles 2 and 6 ($p<0.2$, Wilcoxon rank sum test). Sc and iv administration of rhIL-3 resulted in similar effects on neutrophil counts (day 22 sc: 3.01 ± 1.70 x10^9/l, iv: 3.24 ± 2.21 x10^9/l, $n=16$) and platelet counts (day 22 sc: 318 ± 168 x10^9/l, iv: 333 ± 134 x10^9/l, $n=16$). At the 10 and 15 μg/kg/day dose steps the mean leukocyte count was raised at all time points compared to the control cycles (Figure 1). At 5 μg effects on the leukocyte count on day 15 and on the recovery of the leukocyte count (day 22) were also observed.

RhIL-3 demonstrated an effect on the neutrophil nadir (Figure 2) at the 10 μg/kg/day (day 15 control: 0.58 ± 0.51 x10^9/l; 10 μg: 1.15 ± 1.29 x10^9/l, $p<0.02$). However, this effect at 10 μg could not be demonstrated when neutrophil counts on day 15, expressed as percentage changes from day 1 of each cycle, were compared with the control cycles at the same dose step (Wilcoxon rank sum test). The recovery of the neutrophil counts was hastened at 5, 10, and 15 μg. The neutrophil counts at these doses were higher on day 22 compared to the control cycles. This was also the case for percentage changes from day 1 compared to the control cycles at each dose step (Wilcoxon rank sum test). In addition, the recovery was faster at the 5, 10 and 15 μg dosages compared to the 1 μg dose (Kruskal-Wallis analysis of variance, $p<0.05$). The faster recovery was mainly caused by increases in segmented neutrophils. The neutrophil count on day 29, the day the next chemotherapy course was scheduled, was higher

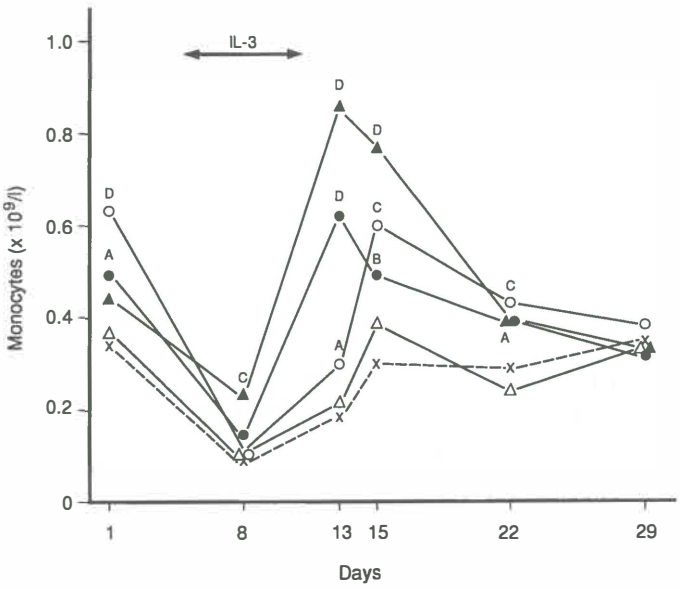

Figure 4. Mean monocyte counts during rhIL-3 administration after chemotherapy.
P values compared to control cycles: a: $p<0.05$, b: $p<0.02$, c: $p<0.01$, d: $p<0.001$.
x = control cycles; ▵ = 1 μg rhIL-3; ○ = 5 μg rhIL-3; ▲ = 10 μg rhIL-3; ● = 15 μg rhIL-3.

at all rhIL-3 doses than in the control cycles. An increase in banded neutrophil counts (Figure 3) was observed on days 8, 13 and 15. More immature myeloid cells other than band forms did not contribute to the difference in neutrophil counts.

Monocyte counts were raised at the 5-15 μg doses (Figure 4). The most pronounced effect was observed at 10 μg rhIL-3. For the eosinophils rhIL-3 demonstrated a dose response effect, with the highest counts on day 13, especially at 15 μg (Figure 5). An effect on the basophil counts was mainly apparent at the 5, 10, and 15 μg dose steps. However, this was probably of little clinical interest, as the basophil counts never exceeded 0.2 $x10^9$/l. Lymphocyte counts were affected at 10 and 15 μg (Figure 6).

The mean platelet count (Figure 7) on day 15, the day of nadir, was raised at the 10 μg dose compared to the control cycles (control: 82.6 ± 60.5 $x10^9$/l; 10 μg: 151.7 ± 99.7 $x10^9$/l, $p<0.01$). However, as for the neutrophil counts, this effect at 10 μg could not be demonstrated when platelet counts on day 15, expressed as percentage changes from day 1 of each cycle, were compared with the control cycles at the same dose step (Wilcoxon rank sum test). At 1 and 5 μg platelet transfusions were necessary in 3 out of 25 cycles with rhIL-3 (1 μg: 1/12 cycles, 5 μg: 2/13 cycles), while no platelet transfusions were necessary at the 10 μg and 15 μg. Platelet transfusions were needed in 7 out of 45 control cycles ($p<0.5$). In addition, the recovery of the platelet count (day 22) was faster at all rhIL-3 doses. This was also the case

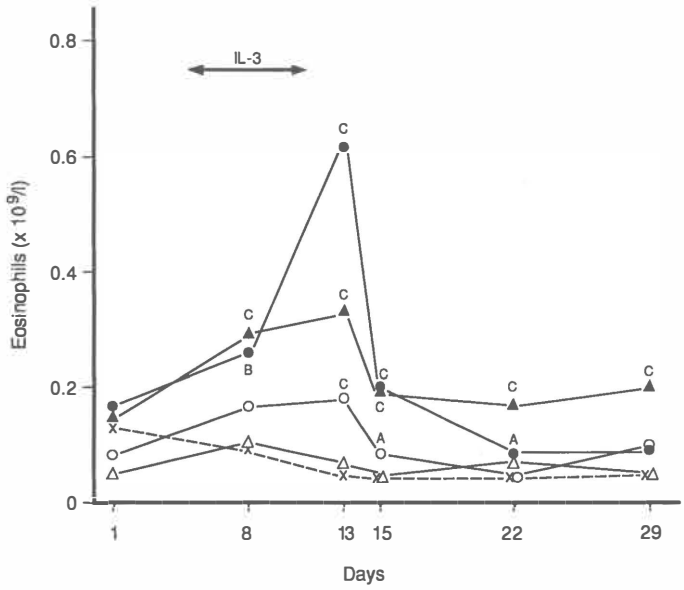

Figure 5. Mean eosinophil counts during rhIL-3 administration after chemotherapy.
P values compared to control cycles: a: $p < 0.02$, b: $p < 0.01$, c: $p < 0.001$.
x = control cycles; ▵ = 1 μg rhIL-3; ○ = 5 μg rhIL-3; ▲ = 10 μg rhIL-3; ● = 15 μg rhIL-3.

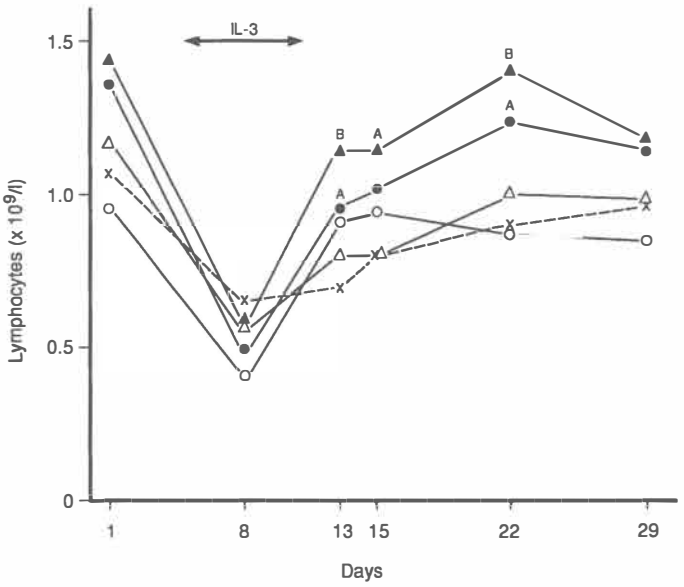

Figure 6. Mean lymphocyte counts during rhIL-3 administration after chemotherapy.
P values compared to control cycles: a: $p < 0.05$, b: $p < 0.01$.
x = control cycles; ▵ = 1 μg rhIL-3; ○ = 5 μg rhIL-3; ▲ = 10 μg rhIL-3; ● = 15 μg rhIL-3.

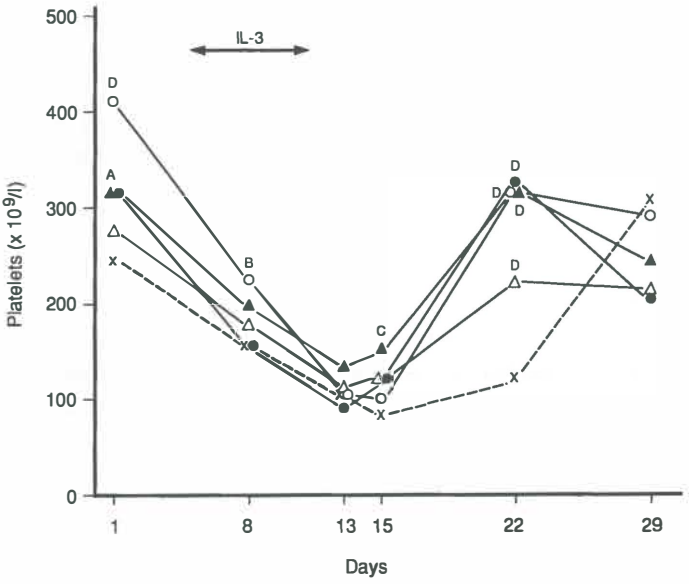

Figure 7. Mean platelet counts during rhIL-3 administration after chemotherapy.
P values compared to control cycles: a: $p<0.05$, b: $p<0.02$, c: $p<0.01$, d: $p<0.001$.
x = control cycles; ▵ = 1 μg rhIL-3; ○ = 5 μg rhIL-3; ▲ = 10 μg rhIL-3; ● = 15 μg rhIL-3.

for percentage changes from day 1 compared to the control cycles at each dose step (Wilcoxon rank sum test). No difference in recovery between the different dose steps could be demonstrated (Kruskal-Wallis analysis of variance).

Reticulocytes were higher at 10 μg (day 15: 18±13 ‰, $p<0.001$) and 15 μg (day 13: 13±14 ‰, $p<0.05$ and day 15: 14±9 ‰, $p<0.01$) rhIL-3 compared to the control cycles (day 13: 6±5 ‰, day 15: 8±5 ‰).

Documented urinary tract infections requiring iv antibiotic treatment occurred in two patients during the first iv administered rhIL-3 cycle (5 and 10 μg/kg). A Herpes Simplex Type I infection was observed during one rhIL-3 cycle (10 μg/kg) and three control cycles. None of the patients was hospitalized because of neutropenic fever in either the rhIL-3 cycles or the control cycles.

Chemotherapy postponement due to prolonged myelotoxicity was necessary in 22 out of 45 control cycles and in 2 out of 49 cycles (one patient not evaluable due to tumor progression in cycle 1) with rhIL-3 ($p < 0.001$, chi-square test). Postponement only was necessary in one patient at 1 μg after cycle 1 and 3. One patient at the 1 μg/kg dose was taken off study after cycle 3 because of insufficient bone marrow recovery. Because of tumor progression three patients (two at 1 μg, one at 5 μg) were taken off study after cycle 5 and one patient (5 μg) after 21 days in cycle 1.

RhIL-3 administration did not result in elevation of IL-6 plasma levels, while slight increases in TNF-α plasma levels only occurred on the last day of rhIL-3 administration at the 5-15 μg/kg doses (median values day 11 cycle 3: 1 μg: 8 ng/l; 5 μg: 19 ng/l; 10 μg: 15 ng/l; 15 μg: 27 ng/l). CRP and SAA plasma levels were only slightly elevated at the 10 μg/kg dose on the last day of rhIL-3 treatment (median value day 11 cycle 3: CRP: 15 mg/l, SAA: 12 mg/l).

Side effects

Fifty-two cycles with rhIL-3 (1 μg/kg: 12 cycles, 5 μg/kg: 13 cycles, 10 μg/kg: 15 cycles, and 15 μg/kg: 12 cycles) were evaluable for toxicity (Table 1). Most frequently observed side effects were fever WHO grade I-II and headache. Both symptoms were rhIL-3 dose-dependent and usually responded to acetaminophen (max. 3 g/day). At 15 μg/kg headache became dose-limiting in two patients during iv administration in cycle 1 and 3, respectively. RhIL-3 administration in these patients was discontinued on days 7 and 8, respectively. Over 50 % of the patients experienced flu-like symptoms at 5-15 μg. A haemorrhagic rash (seven cycles) in the groins, axillas and on the lower extremities usually occurred on days 8 or 9, a time point at which the platelet counts still were above 50 $x10^9$/l in all patients, and disappeared after discontinuation of rhIL-3. Local redness or infiltrates at the sc injection site were noticed in a minority of the cycles (11/32). Thrombophlebitis during iv rhIL-3 occurred in 9/20 cycles, however this was not rhIL-3 dose-dependent. Facial erythema, starting 1-6 hours after rhIL-3 administration and lasting from hours to days, occurred at the 5-15 μg/kg dose steps especially during the first days of treatment and appeared to be dose-dependent in the patients treated iv. One patient at the 5 μg/kg dose experienced flushing in cycles 3 and 5 on the first day of sc administration. Flushing occurred once within 15-30 minutes after rhIL-3 and rapidly disappeared within 10-15 minutes. Peripheral basophil counts and urinary histamine metabolite levels at that time were not elevated in this patient. Moreover, rhIL-3 administration did not raise urinary histamine metabolites levels in any of the patients.

Two patients at the 1 μg/kg/day dose step had elevated liver function enzymes at entry of the study. One experienced both in cycle 1 (with rhIL-3) and cycle 2 (without rhIL-3) reversible liver toxicity WHO grade IV, while the other experienced grade III in both cycles. The first patient refused further treatment with rhIL-3 after cycle 2. At the other doses liver toxicity grade II occurred in five cycles (four at 10 μg and one at 15 μg), while grade III was observed in four cycles (10 μg: 1, 15 μg: 3). In the control cycles grade II occurred in one cycle at 5 μg, while grade III and IV occurred in one cycle at the 10 and 15 μg, respectively. Overall liver toxicity occurred in 11/52 cycles with rhIL-3 versus 5/45 control cycles. Differences in liver toxicity between the different dose steps and the control cycles were not significant. Apart from this and the chemotherapy-related nausea no other toxicity was observed in the control cycles.

Table 1. RhIL-3-related toxicity during sc or iv administration

	IL-3 doses (μg/kg/day)							
	1		5		10		15	
	sc	iv	sc	iv	sc	iv	sc	iv
number of cycles	7	5	8	5	10	5	7	5
Fever WHO gr I-II	1 (14%)	2 (40%)	3 (38%)	5 (100%)	6 (60%)	5 (100%)	7 (100%)	5 (100%)
Headache	-	-	2 (25)	1 (20)	9 (90)	5 (100)	7 (100)	5 (100)[a]
Flu-like symptoms/ chills	3 (43)	-	5 (63)	1 (20)	6 (60)	2 (40)	4 (57)	5 (100)
Flushing	-	-	2 (25)	-	-	-	-	-
Facial erythema	-	-	1 (13)	-	2 (20)	1 (20)	1 (14)	3 (60)
Nausea	1 (14)	-	2 (25)	-	5 (50)	3 (60)	-	3 (60)
Rash	-	-	1 (13)	2 (40)	1 (10)	1 (20)	-	2 (67)[b]
Urticaria	-	-	2 (25)	-	-	-	-	-

[a]: Two patients off study due to severe headache. [b]: Only 3 cycles evaluable for rash.
Incidence of thrombophlebitis during iv rhIL-3 and local reaction after sc rhIL-3 are described in the text.

DISCUSSION

In the present study it was shown that, in patients treated with chemotherapy, rhIL-3 could be safely administered and resulted in a multilineage stimulation of haematopoietic cells. The route of administration was not of influence on hematological effects, as no difference in effect on neutrophil and platelet counts between sc and iv rhIL-3 could be demonstrated. At 15 μg/kg/day uncontrollable headache was dose-limiting. In this respect iv rhIL-3 was less well tolerated than sc administration. The toxicity profile and incidence of the described side effects of sc rhIL-3 after chemotherapy were almost identical to the profile reported by others in patients not treated with chemotherapy (19-21). Liver toxicity appeared to be not clearly related to rhIL-3 administration. Our observation of flushing occurring only after the first rhIL-3 injection and the decreasing frequency of facial erythema during rhIL-3 demonstrates a certain degree of tachyphylaxis. Because urine histamine metabolite levels did not increase during rhIL-3 administration, histamine release from basophils was most probably not the cause of side effects. Preclinical trials and in vitro studies showed a rhIL-3-related histamine release from basophils (28-30), but also other clinical trials demonstrated no increase of histamine release from basophils (19-22). Data on side effects of rhIL-3 administered iv are limited. Ganser et al. (19) observed transient acrocyanosis and chills in all three patients treated with 125 $\mu g/m^2$/day as iv bolus. Kurzrock et al. (22) administered rhIL-3 (30-1000 $\mu g/m^2$/day) by a daily four hour iv infusion for 28 days to patients with bone marrow failure. In their study patients at all dose levels were febrile and headache was dose-related but usually controlled with acetaminophen or codeine. In two patients rhIL-3 at 500 $\mu g/m^2$ was discontinued: because of severe headache, nausea and vomiting in one patient and because of recurrent urticaria in another.

RhIL-3 demonstrated a multilineage haematopoietic response after chemotherapy with increases in leukocytes, platelets and, to a lesser extent, reticulocytes compared to the control cycles. It is possible that the degree of cytopenia in the control cycles was slightly influenced by administration of rhIL-3 in the prior cycle. However, since all control cycles were preceded by a rhIL-3 cycle, such an effect could not be demonstrated. RhIL-3 at doses from 5-15 μg/kg/day demonstrated a clear effect on the neutrophil counts. The increase in neutrophil counts was most eminent during the recovery phase, a week after stopping rhIL-3 treatment. This is of clinical importance as it demonstrates that rhIL-3 can reduce the duration of neutropenia after chemotherapy and could diminish the risk of infectious complications. None of the rhIL-3 dose steps demonstrated a clear effect on the neutrophil nadir counts. The early effects on the neutrophil counts after three days of rhIL-3 treatment at the higher dose steps suggest a mobilization of neutrophils from the bone marrow or marginal pool. A similar early response to rhIL-3 was observed in some patients with aplastic anemia (21). RhIL-3 also increased the monocyte, eosinophil, basophil and lymphocyte counts at the 5-15 μg doses. A similar multilineage effect of rhIL-3 has been demonstrated in previous clinical trials (19-22).

However, we could not demonstrate differences in effects on leukocyte counts between the 5, 10 and 15 μg dose steps.

Of interest is the effect of rhIL-3 on the platelet counts. At all rhIL-3 dose steps platelets recovered faster in comparison with the control cycles. Since there was no significant difference in creatinine clearance between the rhIL-3 dose steps, a comparison of the rhIL-3 effects on the platelet counts at the different dose steps could be made (31). Although no clear protection of platelet nadirs could be demonstrated, there was a tendency towards less platelet transfusions in cycles with rhIL-3. At the 10 and 15 μg/kg doses no platelet transfusions were necessary. However, we could not demonstrate differences in effects on platelet recovery between the different rhIL-3 dose steps. RhIL-3 at doses of 60-500 $\mu g/m^2$/day administered sc to cancer patients with normal hematopoiesis increased the peripheral neutrophil, monocyte, eosinophil, lymphocytes and platelet counts in a dose-related manner (19), while the platelet counts started to increase earlier at higher dosages with counts continuing to rise for one week after discontinuation of rhIL-3. The missing dose-related increase in leukocyte and platelet counts at the higher rhIL-3 dose levels may be related to the small groups of patients or the duration of rhIL-3 administration. In the German study the most pronounced increase in leukocyte and platelet counts occurred in the second week of rhIL-3 treatment (19). Studies with rhIL-3 in patients with bone marrow failure, aplastic anemia or myelodysplastic syndrome seem to demonstrate that the magnitude of effect of rhIL-3 on the leukocyte and platelet counts is closely related to the remaining bone marrow function (19-22).

RhIL-3 can stimulate proliferation of some solid tumor cells in vitro (32-34). In addition, it was recently demonstrated that a small cell lung carcinoma cell line possessed IL-3 receptors (34). However, it appears that CSF's can stimulate in vitro proliferation of only a minority of tumor cell lines and tumors (34-36). Although in our patients there was no evidence for an adverse effect of rhIL-3 on tumor growth, the group was too small to draw any final conclusions concerning this possible effect.

In several clinical studies it was shown that rhGM-CSF induced increases in TNF-α, IL-6, CRP and/or SAA serum levels in vivo (8, 37, 38). Since IL-3 is an earlier acting hematopoietic growth factor than GM-CSF, a similar or more pronounced effect of rhIL-3 on these cytokine plasma levels appeared possible. Effects of rhIL-3 on cytokine plasma levels were limited and indicated that rhIL-3 does not induce an acute phase response in patients treated with chemotherapy. Oster et al. reported slight increases in CRP serum levels after iv bolus rhIL-3 and in some patients slight increases in IL-6 serum levels (39).

The present study demonstrated that rhIL-3 up to doses of 10 μg/kg/day can be safely administered to patients treated with chemotherapy. The MTD in this study was 10 μg/kg/day as severe headache was dose-limiting at 15 μg. Of interest was the similar efficacy of sc and iv rhIL-3 administration as, for a wider application of rhIL-3, use on an outpatient basis favors the sc pathway. RhIL-3 at doses of 5-15 μg/kg demonstrated a multilineage effect on the hematopoiesis. Although both GM-CSF and G-CSF can reduce chemotherapy-related

neutropenia (5-9), the effect of GM-CSF on platelet counts is inconsistent (7-10), while G-CSF does not affect the platelet recovery at all (5, 6). Therefore, rhIL-3 appears to be an interesting CSF for attempting to reduce chemotherapy-related neutropenia and thrombocytopenia, thus probably diminishing the risk of infectious complications and bleeding.

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Chapter 5

PHARMACOKINETICS OF RECOMBINANT HUMAN INTERLEUKIN-3 ADMINISTERED SUBCUTANEOUSLY AND BY CONTINUOUS INTRAVENOUS INFUSION IN PATIENTS AFTER CHEMOTHERAPY FOR OVARIAN CANCER

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SUMMARY

Twenty chemotherapy-naive patients with ovarian carcinoma received 1, 5, 10 or 15 μg/kg/day (five patients per dose step) recombinant human Interleukin-3 (rhIL-3) over seven days after carboplatin/cyclophosphamide in cycles 1 and 3. Patients received rhIL-3 by continuous intravenous (iv) infusion or once daily subcutaneous (sc) injection in cycle 1 and the alternate route in cycle 3. Plasma rhIL-3 samples were obtained once daily on days 1-6 and serially over a 24 h period on day 7 for pharmacokinetic assessment of sc and iv administered rhIL-3 in 16 and 17 patients, respectively. Concentrations were assayed by a time-resolved fluorescence sandwich immunoassay. Pharmacokinetic parameters were derived by noncompartmental methods. Mean steady-state concentrations (C_{ss}) during continuous iv infusion ranged from 117 pg/mL (1 μg/kg/day) to 2217 pg/mL (15 μg/kg/day) and were linearly related to dose ($r=0.87$, $p<0.001$). When dose-normalized, Css was comparable at all doses. The total body clearance (CL) was approximately 4-5 mL/min/kg. Elimination half-life ($t_{1/2iv}$) could be assessed for the 5-15 μg/kg/day dose levels and was 53, 41 and 26 min for the 5, 10 and 15 μg dose levels, respectively (NS between dose levels). Following sc injection, the maximum rhIL-3 plasma concentration ranged from 206 pg/mL (1 μg/kg/day) to 6930 pg/mL (15 μg/kg/day). Both C_{max} ($r=0.89$, $p< 0.0001$) and area under the plasma-concentration time curve ($r=0.93$, $p< 0.0001$) were related to dose. Dose-normalized values were comparable over the entire dose range. Elimination $t_{1/2sc}$ was 4.8 h at the 1 μg dose level and roughly half this time for the 5-15 μg/kg/day dose levels. The systemic clearance of approximately 5-6 mL/min/kg was comparable at all dose levels. Based on trough levels of the seven days sc course, no rhIL-3 accumulation occurred. Bioavailability of sc administered rhIL-3 was nearly 100%. No correlation between

creatinine clearance and pharmacokinetic parameters of rhIL-3 could be demonstrated. Since there was also no difference in hematological efficacy between the two routes of rhIL-3 administration, we conclude that the sc route of administration appears to have no disadvantages over the iv route and may facilitate its clinical application.

INTRODUCTION

Interleukin-3 (IL-3) is a hematopoietic growth factor that promotes the survival, proliferation and differentiation of multipotential hematopoietic stem cells and of the committed progenitor cells of the megakaryocyte, granulocyte/macrophage, erythroid, eosinophil, basophil and mast cell lineages in vitro (1-4). It also affects mature cells by increasing cytotoxic functions or inducing release of cytokines, but does not affect the function of mature neutrophils (5, 6).

Recent clinical studies with recombinant human (rh) IL-3, administered to patients with advanced malignancies, myelodysplastic syndromes, aplastic anemia or bone marrow failure (7-11), demonstrated its multilineage stimulation of hematopoiesis in vivo. These effects were especially prominent in patients with normal bone marrow function (7). Recently we have demonstrated that rhIL-3 administered once daily subcutaneously (sc) or by continuous intravenous (iv) infusion reduced the duration of chemotherapy-induced neutropenia and thrombocytopenia in patients treated for advanced ovarian cancer (12). A similar effect of rhIL-3 has been demonstrated in patients with relapsed small cell lung cancer treated with chemotherapy (13).

To date, the pharmacokinetic parameters of rhIL-3 have been studied in only a limited number of patients (10, 11). In addition, these patients were not being treated with chemotherapy. Therefore, in addition to evaluating the hematological effects and toxicity profiles (12), we studied the pharmacokinetics of rhIL-3 administered at doses of 1-15 μg/kg/day once daily sc and by continuous iv infusion at different doses in patients with ovarian cancer receiving cytotoxic treatment.

MATERIALS AND METHODS

Chemotherapy-naive patients, between 18 and 70 years of age, with Stage III-IV ovarian cancer according to the International Federation of Gynecologists and Obstetricians (FIGO) and eligible for treatment with chemotherapy were entered. Patients with severe heart, lung, liver (serum total bilirubin $\geq$ 40 μmol/L), or kidney impairment (creatinine clearance $\leq$ 50 mL/min) were excluded from the study, as were patients with a WHO performance score grade 3-4. Chemotherapy comprised six courses of carboplatin (300 mg/m^2) and cyclophosphamide (750 mg/m^2), both given iv on day 1 every four weeks on an outpatient basis. Carboplatin

(Bristol-Myers Squibb, Troisdorf, Germany), dissolved in 250 mL 5% dextrose, was infused over 30 min, cyclophosphamide (ASTA Pharma A.G., Frankfurt, Germany), dissolved in 250 mL 0.9% saline, over 15 min.

The patients were divided into four groups of five patients each. After the first, third and fifth chemotherapy course, each group received 1, 5, 10 or 15 μg/kg/day rhIL-3 for seven days (day 5-11), starting four days after chemotherapy. Escherichia Coli derived nonglycosylated rhIL-3 (2-10 x10^6 U/mg protein) was provided by Sandoz (Basle, Switzerland) in vials of 150 μg (for iv administration) and 300 μg (for sc administration). For sc administration rhIL-3 was reconstituted with 1 mL sterile water. If the volume exceeded 4 mL the dosage was divided over both upper legs. For the daily iv infusion polypropylene syringes were used for each of two 12-h infusions, containing half of the daily rhIL-3 dosage, 96 mg (2 mg/mL) human serum albumin and 0.9% saline with a total volume of 48 mL. RhIL-3 in the syringes remained stable for 24 h.

The patients were randomized for either once daily sc or continuous iv rhIL-3 administration in cycle 1. In cycle 3 the alternative route of administration was used. During cycle 5 all patients received sc rhIL-3. Complete blood cell counts were performed on days 1 (before chemotherapy), 8, 13, 15 and 22. Liver function tests were performed on days 1, 8, 15 and 22. Creatinine clearance at entry was calculated from the creatinine levels in 24-h urine and in serum. The patient group in this study was part of the group described in the phase I/II study with rhIL-3 administered to patients on chemotherapy treatment for ovarian cancer (12). The study was approved by the Medical Ethical Committee of the University Hospital of Groningen. Informed consent was obtained from all patients.

Pharmacokinetic assessment of rhIL-3

Pharmacokinetic assessment of rhIL-3 was performed in cycles 1 and 3. Blood samples for pharmacokinetic analysis of sc administered rhIL-3 were drawn prior to the first (0 h), second (24 h), third (48 h), fourth (72 h), sixth (120 h) and seventh (144 h) injection, and following the seventh (and last) injection at 2, 3, 4, 6, 10, 13, 24 and 48 h. Blood samples for pharmacokinetic analysis of rhIL-3 administered by continuous iv infusion were drawn prior to the start of infusion (0 h), and at 24, 48, 72, and 168 h during the infusion. Additional blood samples were taken 0.5, 1, 2, 4, 8, 14, 24 and 36 h after discontinuation of the rhIL-3 infusion. Venous blood samples were collected in 10 mL vacutainer tubes containing sodium heparin (Becton Dickinson, Meylan Cedex, France) and immediately put on ice. Within 30 min after collection the samples were centrifuged at 4 °C and the collected plasma was frozen in polystyrene vials at -20 °C.

The quantitative measurement of rhIL-3 plasma levels was carried out using a time-resolved fluorescence sandwich immunoassay. The assay used was basically as described previously (14), but with a streptavidin-europium conjugate (15). At the appropriate assay dilution, commercially available streptavidin-europium (Wallac Oy, Finland) was found to give

the same results, and was used in the analysis of the samples. Neither of the two monoclonal antibodies used in the sandwich immunoassay showed any cross-reactivity with nine other lymphokines (16). Calibration and quality control samples, containing known amounts of rhIL-3 in normal pooled human plasma and kept stored in aliquots at -20 °C, were analyzed with every batch of patient samples. Calibration curves were constructed and the concentrations of rhIL-3 in both quality control and patient samples interpolated from a logit/log linearization of the calibration samples. The dynamic range of the standard curve was from 40 to 20,000 pg/mL plasma, although no samples were found to have concentrations in the upper range of the curve. The limit of quantification, based on the lowest quality control sample with a coefficient of variation of less than 20%, was 50 pg/mL plasma (n=82). Any systemic bias in measured concentrations was calculated from the results of several quality control samples ranging in concentration from 50 to 15,000 pg/mL plasma. The percent bias (mean-expected concentration/expected concentration) x 100) was found to vary from -2% to +15%. However, the bias measured showed no overall positive or negative trend, with respect to concentration. Three cycles of freezing and thawing of quality control samples at four different concentrations had no deleterious effect on the concentrations of rhIL-3 measured when compared to the value obtained when the samples had been freshly prepared.

The pharmacokinetic characteristics of rhIL-3 were derived by standard noncompartmental methods. The maximum measured plasma concentration (C_{max}) and the time of its occurrence (t_{max}) were compiled directly from the concentration/time data after sc administration. The mean steady-state plasma concentration during continuous iv infusion (C_{ss}) was calculated from the concentration data obtained over the seven-day continuous infusion course. The area under the concentration/time curve (AUC) was calculated by the trapezoidal rule over one dosing interval for the sc injection and for iv administration from the beginning of the continuous infusion to the time (z) of the last measurable plasma concentration (C_z) above the assay limit of quantification with subsequent extrapolation to infinity by the addition of the term C_z/λ_z. The terminal elimination rate constant (λ_z) was derived from linear regression analysis and the corresponding half life ($t_{1/2}$) was calculated as $\ln(2)/\lambda_z$. The apparent systemic clearance after sc administration was calculated as $CL_{sc}/f = Dose/AUC_{sc}$, where f is the bioavailability by this administration route. For the iv data, CL_{iv} was calculated as $CL_{iv} = (k_0T)/AUC_{iv}$, where k_0 is the infusion rate and T is the duration of infusion. For two patient whose post-infusion plasma concentrations were below the limit of quantification (one patient at 1 and 5 μg/kg/day, respectively), CL_{iv} was calculated only from the continuous infusion data as k_0/C_{ss}.

Statistical analysis

To explore dose linearity, C_{ss}, C_{max}, and AUC were modelled as linear functions of dose by linear regression. Analysis of variance was performed to determine the regression coefficient (r) and the significance of the fit. Linear regression was also used to determine possible

relationships between creatinine clearance and pharmacokinetic parameters. In addition, for comparing $t_{1/2}$ by both routes of administration the Wilcoxon rank sum test was used. To evaluate the hematological efficacy of sc and iv rhIL-3 compared to two control cycles (cycles 2 and 4), the two-tailed Student's t-test was used. Only p values < 0.05 were considered significant.

RESULTS

Plasma samples for sc and iv rhIL-3 pharmacokinetic analysis were obtained from 16 and 17 patients, respectively. The median age of the patients in the sc and iv group was 60 and 59 years, respectively (range 21-69). Evaluable for the sc data were four, three, five and four patients at the 1, 5, 10 and 15 μg/kg/day rhIL-3 dose levels, respectively. For the iv data the number of evaluable patients at the different dose steps was four (1 μg), five (5 μg), five (10 μg) and three (15 μg). The other patients were lost for pharmacokinetic evaluation due to premature discontinuation of rhIL-3 administration because of rhIL-3-related side effects, accidental sc infusion of rhIL-3 or tumor progression.

Subcutaneously administered rhIL-3

The predose rhIL-3 plasma concentrations prior to the consecutive rhIL-3 injections were all below the quantification limit for 14 of the 16 patients. For two patients (one at 10 μg/kg/day and one at 15 μg/kg/day) residual concentrations ranging from 110-180 pg/mL were observed. Following the seventh sc injection on the pharmacokinetic profiling day, however, all subjects had a 24 h rhIL-3 plasma concentration below the limit of quantification. Figure 1 shows the median rhIL-3 plasma concentrations following the seventh injection for each rhIL-3 dose level. C_{max} ($r=0.89$, $p<0.0001$) and AUC_{sc} ($r=0.93$, $p<0.0001$) derived from these data demonstrated a rhIL-3 dose-related increase over the entire dose range (Table 1). The median t_{max} was 3.5 h (range 2.0-4.0) for the 1 μg/kg/day rhIL-3 dose and 3.0 h (range 2.0-4.0 h) for the 5, 10 and 15 μg/kg/day rhIL-3 dose levels. The AUC_{sc}/dose was comparable over the entire dose range. The median $t_{1/2sc}$ was 4.8 h at the 1 μg/kg/day dose level and roughly half this value for the three higher dose levels: 2.27, 2.15 and 2.49 h for the 5, 10 and 15 μg dose levels, respectively. An apparent systemic clearance of approximately 5-7 mL/min/kg was comparable at all dose levels. The mean CL_{iv}:CL_{sc}/f ratio, calculated as an estimate of bioavailability after sc administration was 0.97 ± 0.25.

No relationship between creatinine clearance and CL_{sc}/f ($r= -0.17$) was found. In addition, no relationship between creatinine clearance and AUC_{sc}/dose or $t_{1/2sc}$ could be demonstrated ($r= -0.01$, and $r= -0.06$, respectively). Liver function disturbances did not appear to affect the AUC_{sc}/dose or $t_{1/2sc}$, as patients with liver toxicity (WHO criteria) demonstrated similar values for these parameters as patients without liver function disturbances.

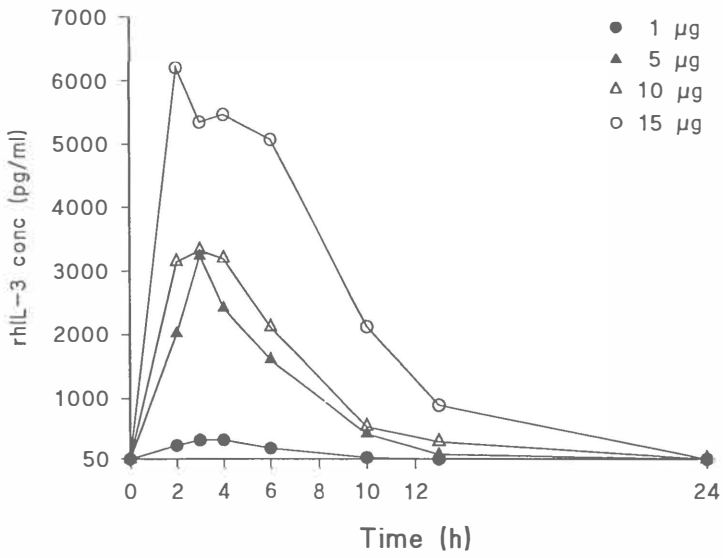

Figure 1. Median rhIL-3 plasma concentrations following the seventh sc rhIL-3 injection.

Continuous iv infusion of rhIL-3

Analysis of the plasma samples of the 17 patients receiving rhIL-3 by continuous infusion yielded 15 pharmacokinetic profiles during the infusion and 13 profiles after stopping the infusion. Plasma concentrations were below the limit of quantification for two patients at 1 µg/kg/day both during and following the end of infusion and for an additional two patients (one at 1 and 5 µg/kg/day each) after stopping the infusion.

The C_{ss} ranged from 117 pg/mL at the 1 µg/kg/day rhIL-3 dose step to 2217 pg/mL at 15 µg/kg/day (Table 2). The relationship of C_{ss} versus dose was adequately described by a linear equation ($r=0.87$, $p<0.0001$). The $t_{1/2iv}$ could be assessed for the 5-15 µg/kg/day rhIL-3 dose levels, and the median values were 53 min at 5 µg/kg/day, 41 min at 10 µg/kg/day and 26 min at 15 µg/kg/day. Although there appeared to be a trend towards a longer $t_{1/2sc}$ compared to the $t_{1/2iv}$, this difference was not significant (Wilcoxon rank sum test for paired observations). CL_{iv} was approximately 4-5 mL/min/kg at all dose levels.

No relationship between creatinine clearance and CL_{iv} ($r= 0.36$), $t_{1/2iv}$ ($r= - 0.32$) or dose-normalized AUC_{iv} ($r= - 0.53$) was found. Also, $t_{1/2iv}$ and dose-normalized AUC_{iv} did not appear to be influenced by liver function disturbances.

In figure 2 the median plasma levels during the 24 hours period after the last sc injection and after discontinuation of the continuous iv infusion in the five patients at 10 µg/kg/day rhIL-3 are demonstrated.

Table 1. Pharmacokinetic parameters of rhIL-3 administered sc after chemotherapy

	rhIL-3 (μg/kg/day)							
	1		5		10		15	
	median	range	median	range	median	range	median	range
C_{max} (pg/ml)	372	206-918	3235	1603-4710	3680	2420-5300	6200	5240-6930
AUC_{sc} (pg.h/ml)	2462	1694-5825	16674	9113-28087	26012	16434-34481	52963	46873-56874
$t_{1/2sc}$ (min)	287	114-392	136	106-136	129	104-206	149	120-217
T_{max} (h)	3.5	2.0-4.0	3.0	2.0-4.0	3.0	2.0-4.0	3.0	2.0-4.0
CL_{sc}/f (ml/min/kg)	7.2	2.9-9.8	5.0	3.0-9.1	6.4	4.8-10.1	4.8	4.4-5.3

Table 2. Pharmacokinetic parameters of rhIL-3 administered by continuous iv infusion after chemotherapy

	rhIL-3 (μg/kg/day)							
	1		5		10		15	
	median	range	median	range	median	range	median	range
C_{ss} (pg/ml)	183	117-249	968	607-1817	1445	952-1554	2214	2058-2217
AUC (pg.h/ml)	18652	---	142108	99873-278417	229968	160303-245154	335342	297997-344256
$t_{1/2iv}$ (min)	53[a]	---	53	43-227	41	22-259	26	24-57
CL_{iv} (ml/min/kg)	4.3	2.8-5.7	4.2	2.1-5.8	5.1	4.8-7.3	5.2	5.1-5.9

[a]: due to concentrations below the limit of quantification, half-life could be calculated for only one subject.

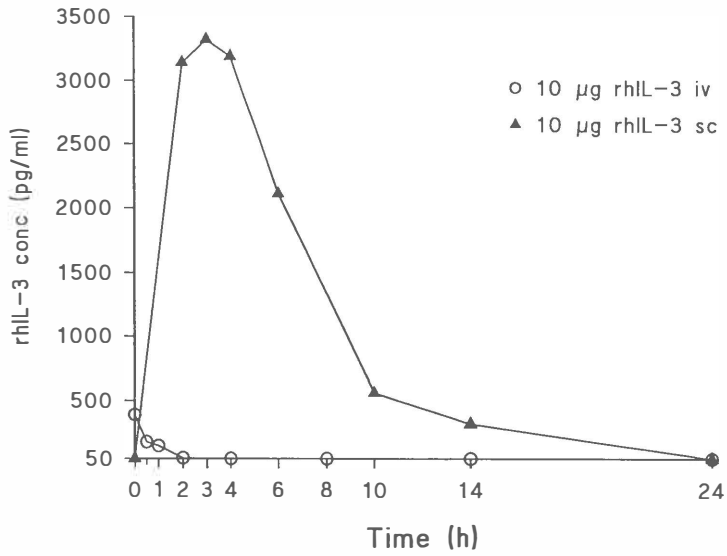

Figure 2. Median rhIL-3 plasma levels during 24 hours after the last sc injection (time 0) and after the discontinuation of the continuous iv infusion (time 0) in five patients treated with 10 µg/kg/day rhIL-3.

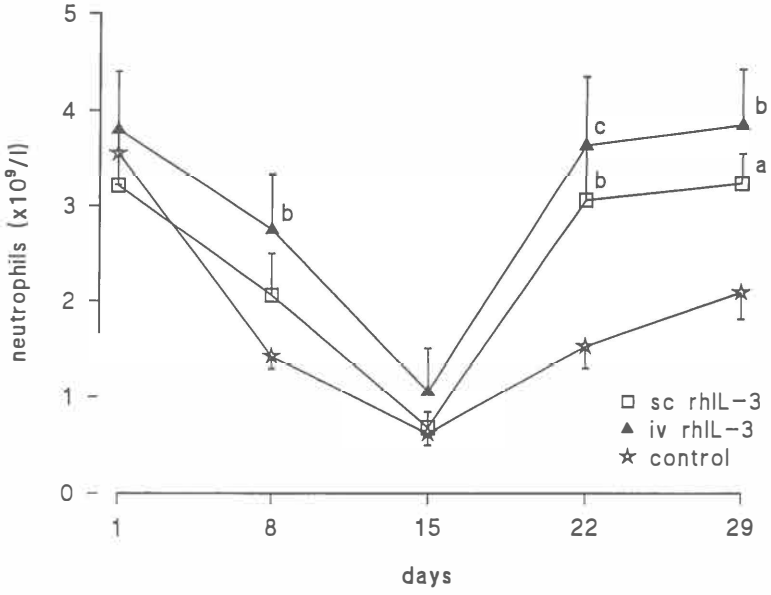

Figure 3. Mean neutrophil counts of patients (n=11) evaluable after both sc and iv administered rhIL-3 in cycles 1 and 3. Curves for sc and iv rhIL-3 represent a summary of all dose levels. Differences in mean neutrophil counts between both routes were not significant. The control curve represents data from cycles 2 and 4 from the 11 patients. P values from the sc or iv rhIL-3 cycles compared to the control cycles: a: $p<0.02$, b: $p<0.01$, c: $p<0.002$. Error bars represent the standard error of the mean.

Effect of route of administration of rhIL-3 on hematological efficacy

To compare hematological efficacy of sc and continuously iv administered rhIL-3, the neutrophil counts during sc and iv administered rhIL-3 in patients evaluable for both sc and iv pharmacokinetics (one patient at 1 μg, two at 5 μg, five at 10 μg and three at 15 μg) were compared on days 1, 8, 13, 15 and 22. (Fig. 3). This was also done for the platelet counts (Fig. 4). No differences in hematological efficacy between the routes of administration on any of the days that blood cell counts were performed was observed. In addition, the hematological efficacy of sc and continuously iv administered rhIL-3 was compared to two control cycles (cycles 2 and 4) of the same group of patients. For the sc and iv route both the neutrophil and platelet recovery was hastened compared to the control cycles.

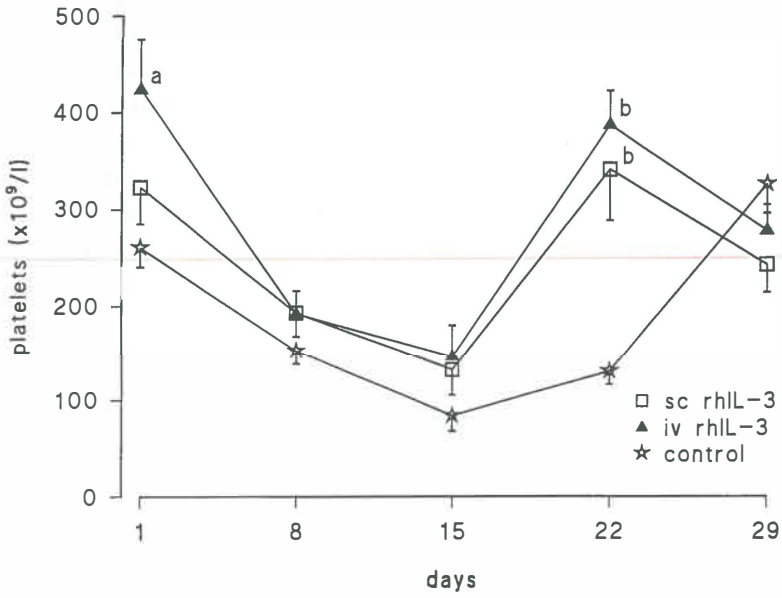

Figure 4. Mean platelet counts of patients (n=11) evaluable after both sc and iv administered rhIL-3 in cycles 1 and 3. Curves for sc and iv rhIL-3 represent a summary of all dose levels. Differences in mean platelet counts between both routes were not significant. The control curve represents data from cycle 2 and 4 from the 11 patients. P values from the sc or iv rhIL-3 cycles compared to the control cycles: a: $p < 0.002$, b: $p < 0.001$. Error bars represent the standard error of the mean.

DISCUSSION

Recently, the importance of the pharmacokinetic properties of rh hematopoietic growth factors has been stressed (17, 18). Limited information, only in patients not treated with chemotherapy, on the pharmacokinetic profile of rhIL-3 administered sc or iv is available (10, 11). This paper

is the first on the pharmacokinetic profile of rhIL-3 administered after chemotherapy. Following once daily sc injection of rhIL-3 in the dose range of 1-15 μg/kg/day no accumulation occurred during the seven day course as assessed by predose concentrations. The difference in the $t_{1/2sc}$ between the 1 μg/kg/day dose step and the three higher dose levels may be due to the small sample numbers and outlier values. Likewise, the apparent deviation from dose-proportionality for C_{max} at 5 μg/kg/day was most likely due to outlier values given the fact that C_{max}/dose was similar for dose steps below and above this level.

Our data indicate that the $t_{1/2iv}$ may decrease with increasing rhIL-3 dose. However, the values of this parameter must be treated as tentative, since retrospectively the sampling strategy was suboptimal for rigorously characterizing the elimination rate. Preliminary conclusions based on the more robust parameter CL_{iv}, which is derived from the entire iv exposure (continuous infusion and post-infusion data), suggest dose-independent elimination over the dose range investigated here. Kurzrock et al. (10) administered rhIL-3 by a daily 4-h infusion at doses of 30-1000 $\mu g/m^2$/day to patients with bone marrow failure. Pharmacokinetic analysis demonstrated a mean $t_{1/2}$ of 18.8 min at the 60 $\mu g/m^2$/day dose step and of 52.9 min at the 250 $\mu g/m^2$/day dose. In patients with advanced malignancies treated with rhIL-3 given as an iv bolus (60 or 123 $\mu g/m^2$/day) or sc (60, 250 or 500 $\mu g/m^2$/day), serum $t_{1/2}$ was 23 ± 3 (mean$\pm$SD) min and 210 ± 15 min, respectively (11). Thus, the $t_{1/2}$'s of rhIL-3 in the present study were in the same range as the $t_{1/2}$'s of other hematopoietic growth factors used in clinical trials (19, 20).

The bioavailability of sc administered rhIL-3 was approximately 100% based on comparative clearance data from the two routes of administration. This is consistent with our observation that the hematological response to rhIL-3 after chemotherapy was equivalent at a given dose regardless of the route of administration (12).

Metcalf et al. (21) reported that in mice injected iv with rIL-3 (1-4 μg), a high proportion of rIL-3 was localized in the liver and the kidney, with the kidney appearing to be an active site of degradation of rIL-3. In 89% of patients with significant renal dysfunction, the clearance of continuously iv infused rhGM-CSF was decreased during the last day of treatment compared to patients with a normal renal function (18). We were unable to relate rhIL-3 pharmacokinetic parameters to the liver function or renal function of patients. However, no patients with very poor liver or renal function were entered in our study.

As reported earlier, rhIL-3-related side effects were dose-dependent (12, 13). Since C_{max}, AUC_{sc} and C_{ss} increased linearly with rhIL-3 dose, a relation between the severity of the side effects and the rhIL-3 dose becomes more likely. Moreover, in our report on the hematological effects of rhIL-3 administered after chemotherapy, there was a tendency towards a dose-response relationship between the administered rhIL-3 dose and the hematological effects. However, the 15 μg/kg/day rhIL-3 dose showed no superior effects over 10 μg/kg/day. In addition, the 15 μg/kg/day dose step demonstrated a more severe toxicity profile. With regard to the linear relationship between the rhIL-3 dose and the AUC_{sc}, this suggests a

biological optimum for sc administered rhIL-3 at approximately 10 μg/kg/day.

In conclusion, sc administered rhIL-3 demonstrated an excellent bioavailability, with a short half-life and equivalent hematological efficacy compared to continuous iv administration. Therefore, the sc route of administration appears to have no disadvantages over the iv route and may facilitate its clinical application.

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Chapter 6

EFFECTS OF HEMATOPOIETIC GROWTH FACTORS ON CHEMOTHERAPY-INDUCED MYELOSUPPRESSION

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I. Introduction

Hematopoietic growth factors are glycoproteins that play an important role in the regulation of the hematopoiesis by affecting the proliferation and differentiation of hematopoietic progenitor cells (Fig. 1). One of the major side effects of chemotherapy treatment is myelosuppression, which apparently cannot be prevented by endogenous production of hematopoietic growth factors. With the cloning of several hematopoietic growth factors the opportunity has been created to test in vivo whether exogenous applied hematopoietic growth factors can sufficiently ameliorate chemotherapy-induced myelosuppression or enhance bone marrow restoration after standard-dose chemotherapy, thus reducing the risk of hemorrhagic and infectious complications. Subsequently, reduced myelosuppression may allow the type of chemotherapy dose-intensification that was previously limited by the degree of myelosuppression. Such chemotherapy dose-intensification without the aid of infusion of hematopoietic progenitor cells, may be achieved by increasing the chemotherapy dose and/or by reducing the intervals between consecutive chemotherapy courses. After ablative chemotherapy followed by bone marrow transplantation (BMT) or reinfusion of peripheral harvested stem cells, hematopoietic growth factors may accelerate reconstitution of the hematopoiesis. Hopefully, chemotherapy dose-intensification will, in the long run, result in increased survival in selected cancer patients, when compared to less intensive, standard regimens in randomized trials.

Here we will discuss the role of hematopoietic growth factors in standard dose chemotherapy regimens and in chemotherapy dose-intensification, including ablative chemotherapy followed by bone marrow rescue.

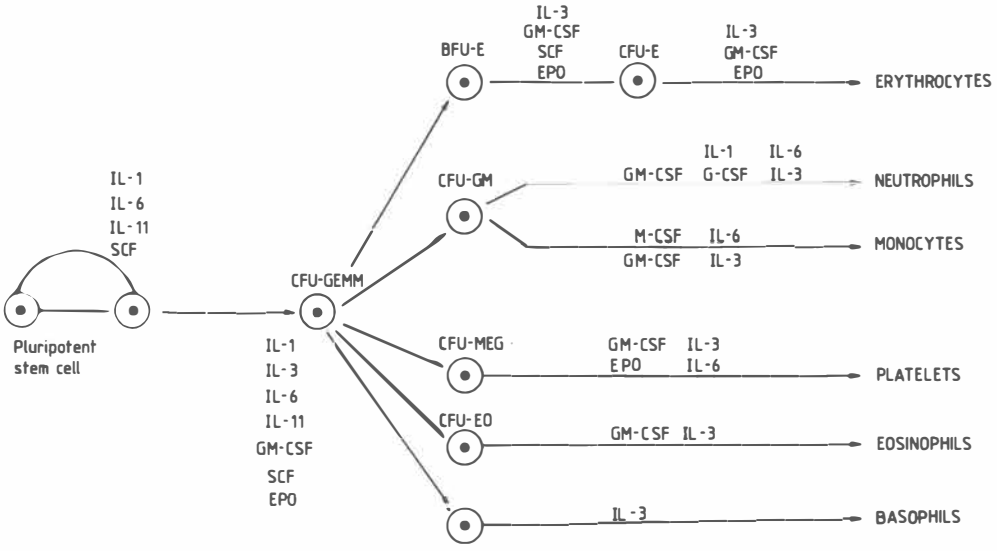

Figure 1. Effects of the rh hematopoietic growth factors discussed in this paper on the hematopoiesis. CFU-GEMM = colony-formingunit granulocyte-erythrocyte-monocyte-megakaryocyte; BFU-E = burst-forming unit erythroid; CFU-E = colony-forming unit erythroid; CFU-GM = colony-forming unit granulocyte-monocyte; CFU-MEG = colony-forming unit megakaryocyte; CFU-EO = colony-forming unit eosinophil.

II. Granulocyte colony-stimulating factor

II-A. Biology

Following the identification and purification of murine Granulocyte colony-stimulating factor (G-CSF) (1), human G-CSF was first purified from a medium conditioned by a bladder carcinoma cell line (2). The gene encoding for G-CSF is located on the long arm of chromosome 17 (3). In vivo, it is produced by monocytes, fibroblasts and endothelial cells (4-7). G-CSF is a lineage-specific regulator of neutropoiesis as it affects differentiation and proliferation of myeloid progenitor cells (8, 9). It affects immature myeloid progenitors and mature neutrophils by enhancing chemotaxis, phagocytosis and microbial killing at infection and inflammatory sites (10-12). G-CSF has been cloned and expressed in E.Coli, yeast and mammalian cells (8, 13, 14). Since the introduction of recombinant human (rh) G-CSF in 1987, the nonglycosylated E.Coli derived rhG-CSF, with a molecular weight (MW) of 19.6 kD, has been predominantly used in clinical trials.

II-B. Animal studies

Several animal studies demonstrated for rhG-CSF a dose-dependent increase in peripheral neutrophil counts at doses up to 500 μg/kg/day administered subcutaneously (sc) (15, 16). In cynomolgus monkeys treated with high-dose cyclophosphamide, rhG-CSF shortened the period of neutropenia (16). A similar reduction in duration of neutropenia by rhG-CSF was demonstrated in primates treated with total-body irradiation (TBI) with or without BMT (17-20).

II-C. RhG-CSF and standard-dose chemotherapy

In the first phase I/II trial in humans, Bronchud et al. treated patients with small cell lung cancer with rhG-CSF prior to and after chemotherapy (21). In the pre-chemotherapy phase, rhG-CSF (1-40 μg/kg/day) was administered by continuous iv infusion for 5 days. A maximum tolerated dose (MTD) was not reached as rhG-CSF-related toxicity was not encountered. RhG-CSF induced an increase in peripheral neutrophil counts at all dose steps. Although no clear dose-response effect in this small group of patients (n=12) could be demonstrated, in general higher neutrophil counts were reached at higher rhG-CSF dosages. In one patient at 10 μg/kg the neutrophil count increased rapidly to 100 x10^9/l, necessitating rhG-CSF dose reduction. No effect of rhG-CSF on other hematopoietic lineages was noticed. Subsequently patients were treated with six cycles of chemotherapy (ifosfamide-mesna and doxorubicin on day 1 and etoposide on days 1-3) every three weeks. RhG-CSF was administered for 14 days by continuous iv infusion every other chemotherapy cycle at the same doses as in the pre-chemotherapy phase, starting 24 h after the last etoposide infusion. Each patient served as his own control. RhG-CSF hastened the recovery of neutrophils at all doses, with differences in neutrophil counts between rhG-CSF cycles and control cycles being significant by day 15. The duration of neutropenia $<$ 1 x10^9/l was shortened by a median of 80%. During the cycles with rhG-CSF support less infectious complications occurred. It was also demonstrated that after in vivo stimulation with rhG-CSF the neutrophils preserved in vitro normal mobility and bactericidal activity (22). Due to a small number of patients, and only two patients at each dose step, the most desirable rhG-CSF dose step could not be determined. Gabrilove et al. treated 27 patients with transitional-cell carcinoma of the urothelium in a phase I/II study with rhG-CSF before and/or after a first chemotherapy course at doses of 1-60 μg/kg/day (23). RhG-CSF was infused iv once daily over 30 minutes (min). In the pre-chemotherapy phase rhG-CSF, administered for six days, induced a dose-dependent increase in the neutrophil count. After the first chemotherapy course rhG-CSF, given for eight days, clearly increased the neutrophil count compared to the following control cycle. This reduced the duration of neutropenia ($<$ 1 x10^9/l) and reduced the number of days on which antibiotics for febrile neutropenia were used. Patients who were previously treated with pelvic radiotherapy performed worse than patients treated with chemotherapy only. Interestingly, both the incidence and severity of mucositis were reduced in the rhG-CSF group. In addition, a higher percentage of patients did receive the next

chemotherapy cycle on time after rhG-CSF than after chemotherapy only. At 60 μg/kg rhG-CSF, an increased monocyte count after chemotherapy was observed in three out of five patients. No effect on other hematopoietic cell lineages was noted. RhG-CSF-related side effects remained restricted to transient bone pain in 72% of the patients before and in 20% after chemotherapy.

In 15 cancer patients treated with melphalan (25 mg/m^2) preceded and followed by rhG-CSF administered twice daily as a 30 min infusion, a similar dose-dependent increase of neutrophil counts was found (24). In the pre-chemotherapy phase rhG-CSF was administered at doses of 1-60 μg/kg/day for five days. After chemotherapy rhG-CSF was administered at doses of 3-60 μg/kg/day for nine days, starting the day after melphalan infusion. In the pre-chemotherapy phase, neutrophil counts up to 80 x10^9/l at the 60 μg/kg dose were reached. At high rhG-CSF doses monocyte and lymphocyte counts increased, while myeloid precursor cells appeared in the peripheral blood. After chemotherapy, the neutrophil nadir did occur earlier and the duration of neutropenia was seven days shorter with high doses of rhG-CSF in comparison with low doses. The increase in neutrophil counts during rhG-CSF administration was less pronounced in patients heavily pretreated with chemotherapy. Again the only clinical side effect of rhG-CSF was slight bone pain.

Morstyn et al. administered once daily sc boluses of rhG-CSF at doses of 0.3, 1 or 3 μg/kg/day to nine (three per dose step) not heavily pretreated patients prior and following melphalan (25 mg/m^2) (25). Following this, two other groups of three patients received rhG-CSF by continuous sc infusion at doses of 3 or 10 μg/kg/day prior and following melphalan. Prior to chemotherapy an increase in peripheral neutrophil counts within 24 hours, followed by an increase in myelocytes and promyelocytes was observed. An increase in monocytes was also observed. Following melphalan, a dose of 10 μg/kg/day rhG-CSF by continuous sc infusion was found to reliably abrogate the neutropenia. In 15 additional patients the efficacy of five different administration schedules of continuous sc infused rhG-CSF (10 μg/kg/day) was studied. It was shown that in this study pre-chemotherapy administration of rhG-CSF did not affect the neutrophil nadir after melphalan and that commencing rhG-CSF treatment eight days after melphalan sufficiently abrogated neutropenia in not heavily pretreated patients. Bone pain was the only clinically relevant side effect of sc administered rhG-CSF. Yoshida et al. treated 16 non-Hodgkin's lymphoma patients with rhG-CSF (5-400 μg/m^2/day) by a 30 min iv infusion for 14 days, starting three days after combination chemotherapy (26). At rhG-CSF doses of more than 100 μg/m^2 the duration of neutropenia was shortened compared to a previous control cycle without rhG-CSF. Also, the duration of fever was reduced in the cycles with rhG-CSF. A dose-dependent increase in neutrophil nadir counts and a dose-dependent shortening of the duration of neutropenia by rhG-CSF (50-800 μg/m^2/day) following intensive chemotherapy was also demonstrated in 24 lung cancer patients (27). Chemotherapy-pretreated patients were less responsive to rhG-CSF than chemotherapy-naive patients. Neidhart et al. treated patients with refractory malignancies with 5-weekly repeated courses of dose-intensive chemotherapy

consisting of cisplatin (150 mg/m^2), etoposide (1.5 g/m^2) and cyclophosphamide (5 g/m^2) (28). In the first cycle ten patients additionally received rhG-CSF at doses of 20, 40 or 60 μg/kg/day as a 30 min iv infusion starting three days after chemotherapy. A concurrent but non-randomized control group for cycle 1 consisted of 11 patients. RhG-CSF at 60 μg/kg shortened the duration of severe neutropenia (< 0.1 x10^9/l) by three days. A neutrophil count of 0.5 x10^9/l was reached four days earlier in the rhG-CSF group. The duration of antibiotic treatment was also shortened by rhG-CSF, but not the duration of hospitalization. In addition to a swifter neutrophil recovery, rhG-CSF also increased the degree of recovery. The effect of rhG-CSF on the neutrophil counts was dose-dependent, while no effect on platelet counts was found.

In contrast to other hematopoietic growth factors, several large randomized studies with rhG-CSF after standard chemotherapy without BMT have been conducted. In the first large randomized trial with 108 patients with relapsed or refractory acute leukemia, rhG-CSF accelerated the recovery of neutrophils after intensive chemotherapy (29). RhG-CSF (200 μg/m^2/day) was administered daily iv over 30 min until the neutrophil count rose above 1.5 x10^9/l. The incidence of documented infections was lower in the rhG-CSF treated patients, although there was no difference in the incidence of febrile episodes.

In a multicentre randomized placebo-controlled trial with 211 newly-diagnosed small cell lung cancer (SCLC) patients, rhG-CSF or placebo (230 μg/m^2/day) was administered sc for up to 14 days after each of a maximum of 6 chemotherapy cycles, scheduled for every three weeks (30). If patients experienced neutropenic fever during a cycle, they were withdrawn from the double-blind study and allowed to receive open-label rhG-CSF for the duration of their treatment. During the first cycle neutropenic fever occurred in 28% of the rhG-CSF patients and in 57% of the placebo patients. When all cycles were taken into account, at least one period of neutropenic fever was encountered by 40% of the rhG-CSF treated patients (cross-over patients not included) and by 77% of the placebo group. In cycle 1 neutropenia (<0.5 x10^9/l) occurred in 84% of the rhG-CSF patients versus 98% of the placebo patients (p=0.001). The duration of this neutropenia in cycle 1 was reduced from 6 days to 3 days by rhG-CSF. In addition, the neutrophil nadir occurred earlier and was less deep (0.07 x10^9/l versus 0.04 x10^9/l) in the rhG-CSF group. The mean number of days of iv antibiotic use was reduced by 47% and the mean number of days of hospitalization by 45% for the rhG-CSF group, as compared with the placebo group, during cycles with blinding. The duration of individual episodes of antibiotic use and hospitalization, however, was similar in both groups. RhG-CSF did not influence the median patient survival time as it was similar for both groups. RhG-CSF caused bone pain in 20% of the patients. In a very similar randomized placebo-controlled study 103 chemotherapy-naive SCLC patients were given rhG-CSF or placebo, starting the day after the last chemotherapy infusion (31). RhG-CSF or placebo (230 μg/m^2/day) was administered sc for up to 14 days. Febrile neutropenia occurred in the rhG-CSF group and placebo group in 26% and 48% of the patients, respectively, while iv antibiotics were needed in 29% and 46%. The duration of hospitalization was shorter in the

rhG-CSF group. Infection-related deaths occurred in none of the rhG-CSF patients and in 6% of the placebo-treated patients. In a phase III trial rhG-CSF was reported to reduce the incidence of serious infectious episodes by 10% in patients with non-Hodgkin's lymphoma treated with chemotherapy (32). KW 2228, a mutant E.Coli-derived rhG-CSF (33) reduced the duration of neutropenia ($< 1 \times 10^9/l$) after chemotherapy for non-Hodgkin's lymphoma (34). Less infectious episodes occurred in the cycles with KW 2228 (34%) than in the control cycles (58%). The optimal dosage of this new compound appeared to be 1 μg/kg/day administered sc. Side effects consisted of asthenia and bone pain. These trials demonstrated that rhG-CSF administered iv or sc can induce a dose-dependent increase in neutrophil counts and that this effect was sustained throughout the rhG-CSF administration. Following chemotherapy, rhG-CSF demonstrated ability to reduce chemotherapy-related neutropenia in a dose-related manner. RhG-CSF administration reduced the incidence of febrile neutropenia and consequently diminished the necessity for iv antibiotic treatment and hospitalization. However, in the large randomized trials (30, 31) only 20-30% of the patients seemed to benefit from rhG-CSF treatment. Increases in the survival time of rhG-CSF treated patients were not reported (30). As both iv (continuous or 30 min infusion) and sc (continuous or bolus) administration have demonstrated a stimulating effect on granulopoiesis, the sc route should be preferred from a practical point of view as it allows rhG-CSF treatment on an outpatient basis. In view of this, it is of importance that the mild toxicity profile of rhG-CSF allows its use on an outpatient basis. Scheduling of rhG-CSF treatment seems essential for the efficacy, as there is a certain lag-phase in the rhG-CSF induced neutrophil response. This implies that commencing rhG-CSF treatment shortly after the last chemotherapy administration seems recommendable, although the optimal starting time point probably also depends on the kinetic properties of the cytotoxic drugs used. Starting rhG-CSF treatment concomitantly with chemotherapy is not recommended, as this may lead to enhanced myelotoxicity (35). With regard to the moment at which administration of rhG-CSF should be discontinued, attempting to reach a neutrophil count higher than a normal count of approximately $5 \times 10^9/l$ may have little extra clinical benefits. The two placebo-controlled studies in SCLC patients (30, 31) demonstrate that a dose of 5 μg/kg given once daily sc can be recommended as a starting dose.

II-D. RhG-CSF and chemotherapy dose-intensification

II-D1. Without bone marrow transplantation

In some phase I/II studies it was shown that rhG-CSF might allow chemotherapy dose-intensification by less chemotherapy postponement (23), less dose reduction (31) or by increasing the chemotherapy doses (26).

In some studies chemotherapy dose intensification has been attempted. Patients with advanced breast cancer or recurrent ovarian cancer were treated with doxorubicin at doses of 75, 100, 125 or 150 mg/m^2 followed by 11 days of rhG-CSF as a continuous iv infusion (36).

The initial rhG-CSF dose was 10 μg/kg/day starting the day after chemotherapy. After one week the dose was reduced to 5 μg/kg/day. Serving as a control group were four patients treated with 75 mg/m^2 doxorubicin without rhG-CSF. Chemotherapy was scheduled to be given every two weeks for up to three cycles. When combined with rhG-CSF, doxorubicin at all dose levels could be given every two weeks. The control patients could only be treated every three weeks, as a neutrophil count $>$ 2.5 x10^9/l was not reached until day 19-21. The neutrophil nadir was reached five days earlier in the rhG-CSF treated patients. In this study rhG-CSF allowed dose intensification by increasing the doxorubicin dose and by reducing the time intervals. However, with increasing chemotherapy doses, more platelet and blood transfusions were required. Corresponding with deeper and longer lasting neutrophil nadirs at doses of 125 and 150 mg/m^2 doxorubicin compared to 75 and 100 mg/m^2, was the need for iv antibiotics in seven out of eight patients at the two higher dose steps. Furthermore, mucositis, occurring at all doses, became more severe with increasing doses of doxorubicin.

Preliminary data from other studies in which rhG-CSF was used to enable chemotherapy dose-intensification in non-SCLC (37), SCLC (38, 39), non-Hodgkin's lymphoma (40, 41) and breast cancer (42, 43) give further support to a role for rhG-CSF as it reduced especially neutropenia duration. However, as expected, thrombocytopenia may remain a dose-limiting factor in chemotherapy dose-intensification without bone marrow support or an additional hematopoietic growth factor.

II-D2. With bone marrow transplantation

Reinfusion of autologous and allogeneic bone marrow is increasingly applied in an attempt to overcome dose-limiting myelosuppression after ablative chemotherapy. In 1989 it was demonstrated by Sheridan et al. that rhG-CSF following ablative chemotherapy and autologous BMT could reduce the period of severe neutropenia (44). They administered rhG-CSF at an initial dose of 20 μg/kg/day by continuous sc infusion to 15 patients with non-myeloid malignancies, starting the day after bone marrow reinfusion. The neutrophil recovery to $>$ 0.5 x10^9/l was accelerated by nine days in comparison with a historical control group. This resulted in a shortening of parenteral antibiotic treatment by seven days. In addition, the period of parenteral nutrition due to severe mucositis was shortened. The duration of reverse-barrier nursing was shortened by eight days. Granulopoiesis in the bone marrow was increased in the rhG-CSF treated patients. RhG-CSF did not affect the platelet recovery. RhG-CSF was well tolerated and bone pain, as described in phase I/II trials without ablative chemotherapy and BMT, was not reported.

In a phase II trial, 18 patients with Hodgkin's disease were treated with an initial rhG-CSF dose of 60 μg/kg/day, administered as a 30 min iv infusion following autologous BMT (45). Again the neutrophil recovery was hastened in comparison with a historical control group. A granulocyte count of 0.1 x10^9/l and 0.5 x10^9/l was reached 4 and 9 days earlier, respectively, in the rhG-CSF treated patients. Platelet recovery was not accelerated by rhG-CSF

administration. The frequency of febrile episodes was not altered by rhG-CSF, since these episodes frequently commenced at a neutrophil count $< 0.1 \times 10^9$/l. Only myalgia and bone pain were reported as rhG-CSF-related side effects at this relatively high dose. Although the study design was different, especially the chemotherapeutic regimens, the previous two studies (44, 45) seemed to demonstrate that 60 μg/kg/day rhG-CSF was not more effective in accelerating the neutrophil recovery than 20 μg/kg/day after autologous BMT. A study with doses of rhG-CSF ranging from 200-800 $\mu g/m^2$/day given for 14 days to 34 patients after allogeneic BMT for leukemia or aplastic anemia did not demonstrate a difference in neutrophil recovery between the different dose steps, although there was a trend towards a faster neutrophil recovery at higher rhG-CSF dosages (46). However, only two patients were treated at the highest rhG-CSF dose.

Until now, these are the three fully published studies in which rhG-CSF was used after BMT in a considerable number of patients. In a phase I/II trial in seven patients after allogeneic BMT for leukemia, the neutrophil recovery was also accelerated by rhG-CSF (50-200 $\mu g/m^2$/day) (47). An ongoing study conducted in patients with high risk lymphoma treated with autologous BMT gives support to the assumption that 20 μg/kg/day rhG-CSF may be the maximum effective dose required (48). In six patients treated with rhG-CSF by continuous sc infusion, a granulocyte count $> 0.5 \times 10^9$/l was reached after a mean of 10.8 days after marrow reinfusion in comparison with 22.5 days in a historical control group of 27 patients. The mean duration of hospitalization was 23.8 days for rhG-CSF treated patients compared to 33.6 days in the historical control group. These studies provide evidence that rhG-CSF can reduce the duration of neutropenia after BMT and thus the risk of infectious complications. However, large comparative studies have not yet been performed, implicating that the value of rhG-CSF in a BMT setting, with regard to hematological reconstitution and its effect on infectious complications and subsequently on the frequency and duration of antibiotic treatment and hospitalization, has yet to defined. Also, the optimum rhG-CSF dose in this setting has not yet been fully established but should most probably be in the range of 5-20 μg/kg/day.

III. Granulocyte-macrophage colony-stimulating factor

III-A. Biology

Human Granulocyte-macrophage CSF (GM-CSF) was purified from conditioned medium of a virus-transformed T-lymphoblast cell line (49). The gene encoding for GM-CSF is located on the long arm of chromosome 5 (50). In vivo, GM-CSF is produced by activated T-cells (50), monocytes (5), fibroblasts (6) and endothelial cells (51). In vitro, GM-CSF acts as a multilineage hematopoietic growth factor on early and later stages of myelopoiesis (9, 52, 53). In addition, GM-CSF induces megakaryocyte precursors either alone (54) or in combination with IL-3 (55) or IL-6 (56). GM-CSF enhances the functional activities of neutrophils, such

as phagocytic activity (57), chemotaxis (58), superoxide production (59) and antibody-dependent cellular cytotoxicity (60). Moreover, GM-CSF induces monocyte cytotoxicity and cytokine release in vitro (61, 62). GM-CSF has also demonstrated ability to induce cytokine release in vivo (63-65). Following cloning (50), GM-CSF was expressed in mammalian cells (50), yeast (66) and bacteria (67). The different types of rhGM-CSF have a MW ranging from 14 to 35 kD, depending on the glycosylation pattern.

III-B. Animal studies

In 1986 Donahue et al. reported a dramatic increase in the peripheral leukocyte counts of healthy monkeys during continuous iv infusion of rhGM-CSF (68). A rhGM-CSF-induced leukocytosis was also demonstrated during sc (three times daily) and, to a lesser extent, during 6-hour iv infusions in rhesus monkeys (69). In primates treated with ablative TBI followed by autologous BMT, rhGM-CSF accelerated the recovery of neutrophils and platelets (70, 71). RhGM-CSF also showed efficacy in restoring hematopoiesis after inadequate engraftment (70).

III-C. RhGM-CSF and standard dose chemotherapy

Shortly thereafter, phase I studies in humans with rhGM-CSF showed a dose-dependent increase in peripheral leukocyte and neutrophil counts (72, 73), Antman et al. were the first to demonstrate efficacy of rhGM-CSF in chemotherapy-induced myelosuppression (74). Sixteen chemotherapy-naive patients with sarcomas were treated with rhGM-CSF (4-64 μg/kg/day continuous iv) before and after the first cycle of chemotherapy. A second control cycle of chemotherapy was not followed by rhGM-CSF. In the pre-chemotherapy phase rhGM-CSF, administered for up to seven days, elevated leukocyte and neutrophil counts in all patients. Surprisingly, the platelet count decreased during the first four days. Twelve patients completed both chemotherapy cycles. In the cycle with a 10-day course of rhGM-CSF, the neutrophil recovery to 0.5 $x10^9$/l was four days earlier than in the control cycle ($p < 0.01$). In addition, the period of neutropenia (< 0.5 $x10^9$/l) was shorter in the rhGM-CSF cycle. In contrast to the pre-chemotherapy data, the platelet nadir count was higher in the rhGM-CSF cycle compared to the control cycle. A dose-response effect of rhGM-CSF could not be demonstrated. Although it could be argued that progressive myelotoxicity accounted for at least in part for the differences in hematological values, the authors found no progressive myelotoxicity in 111 patients previously treated with the same chemotherapy regimen (doxorubicin, ifosfamide, dacarbazine, mesna). RhGM-CSF was well tolerated at doses up to 32 μg/kg/day with fever, arthralgias and mild transient headache as the main side effects. At 64 μg/kg/day fluid retention and thrombus formation around the central venous catheter developed in two out of four patients. Since then numerous phase I/II studies have been performed with rhGM-CSF following chemotherapy.

In the first placebo-controlled double-blind randomized trial with rhGM-CSF after standard dose chemotherapy, we treated 15 patients with ovarian cancer with 1.5, 3 or 6

μg/kg/day rhGM-CSF administered sc once daily for seven days (65). The patients received six cycles of carboplatin (300 mg/m^2) and cyclophosphamide (750 mg/m^2) on day 1 every four weeks followed by rhGM-CSF or placebo on day 6. A neutrophil nadir count $< 0.5 \times 10^9$/l occurred in 29% of the cycles at 6 μg/kg rhGM-CSF compared to 91 % of the placebo cycles ($p < 0.0005$). The neutrophil recovery was also accelerated at this rhGM-CSF dose. Infectious complications were scarce with one patient in the placebo group experiencing a lower urinary tract infection. Furthermore, at the 3 and 6 μg/kg rhGM-CSF dose steps, nadir platelet counts were higher and platelet recovery was faster in comparison with the placebo cycles. Chemotherapy dose-reduction or postponement due to myelotoxicity was necessary in 11% of the rhGM-CSF cycles and 32% of the placebo cycles (NS). RhGM-CSF related side effects remained restricted to local infiltrates at the injection site, necessitating withdrawal from the study of two patients. As previously described (75), capillary leakage of ^{131}I-albumin was increased after chemotherapy, but rhGM-CSF at doses up to 6 μg/kg/day did not further enhance leakage.

Gianni et al. treated 15 patients with breast cancer or non-Hodgkin's lymphoma with high-dose cyclophosphamide (7 g/m^2) followed by 5 μg/kg/day rhGM-CSF, continuously iv infused for 14 days (76). RhGM-CSF treatment was started on the day after chemotherapy and results were compared with a control group of 21 patients. In the rhGM-CSF group, the recovery of the neutrophil count to over 0.5 and 1.0 $\times 10^9$/l was reached six days earlier than in the control group ($p < 0.01$). However, the faster neutrophil recovery did not result in a significant reduction of the infection rate. The duration of thrombocytopenia $< 25 \times 10^9$/l was also reduced in the rhGM-CSF group (0.33 versus 0.7 days for controls, $p = 0.012$), as was the time to achieve a platelet count of 100×10^9/l (14 versus 16 days, $p = 0.004$). As a consequence, there was a trend towards less need of prophylactic platelet transfusions in the rhGM-CSF group (13% versus 43%). The dose of 5 μg/kg/day was in general well tolerated with only one patient requiring discontinuation of rhGM-CSF treatment on the last day of administration due to severe bone and muscle pain. A similar reduction by 5 days in the duration of neutropenia ($< 0.5 \times 10^9$/l) was achieved by rhGM-CSF administration (250 μg/m^2/day continuous iv) after chemotherapy in 23 patients with refractory non-Hodgkin's lymphoma compared to 14 controls (77). The infection rate was not significantly reduced in the rhGM-CSF group (25% versus 53% in controls), but rhGM-CSF treated patients experienced less mucositis (17% versus 60%). In this study no effect on platelet counts was observed. In contrast to other studies, rhGM-CSF at this relatively modest dose demonstrated quite considerable side effects consisting of pleural and pericardial effusions in five patients, and a respiratory distress syndrome in one patient. In addition to a higher neutrophil nadir and an accelerated neutrophil recovery, rhGM-CSF, administered sc for 10 days at a dose of 250 μg/m^2/day to 19 patients with solid tumors or lymphoid malignancies, reduced the duration of parenteral antibiotic treatment after high-dose chemotherapy (78). Results were compared with a previous control cycle in which no rhGM-CSF was given. This to exclude the possibility of progressive myelotoxicity. On the

other hand, this may have negatively influenced the efficacy of rhGM-CSF as the effects of growth factors on myelopoiesis seems related to the remaining bone marrow function (79). The number of days with fever >38.5 °C was also reduced in the rhGM-CSF cycles, as was the incidence and severity of mucositis. However, this did not reduce the duration of hospitalization (30.8 ± 6.4 days versus 37.6 ± 12 days for control cycles, NS). Again, no effect on the platelet counts was observed.

In a randomized, double-blind, placebo-controlled trial we treated 30 patients with chemotherapy-induced leukopenia ($< 1.0 \times 10^9$/l) and fever (≥ 38.5 °C) with seven days of rhGM-CSF (2.8 μg/kg/day continuous iv) or placebo and iv antibiotics (80). Patients entered after standard-dose chemotherapeutic regimens and treated with rhGM-CSF demonstrated an accelerated recovery of the neutrophil counts, but this did not result in a shortening of the period of antibiotic treatment or fever. Presently a large multicentre placebo-controlled randomized trial has been initiated to further assess the efficacy of rhGM-CSF in patients with neutropenic fever. Studies in similar patients with rhG-CSF are in progress, but data are not yet available.

These and other studies (79, 81) demonstrated that rhGM-CSF at doses of approximately 3 to 10 μg/kg/day, administered either by continuous iv infusion or sc, could accelerate the neutrophil recovery after chemotherapy at the cost of in general only mild side effects. The effect on the platelet count was very variable, as some studies showed no effect (77, 78, 80), while others showed elevated platelet counts when rhGM-CSF was given (65, 74, 76, 81, 82). Interestingly, a study conducted by Furman et al. demonstrated that children tolerated rhGM-CSF up to doses of 1500 μg/m^2/day extremely well (83). In fact, 1500 μg/m^2/day did not become the MTD. In their study a dose-dependent increase in neutrophil and platelet counts was demonstrated during rhGM-CSF treatment following chemotherapy, with a most effective dose of approximately 750 μg/m^2/day.

Although some studies demonstrated clinically beneficial effects of rhGM-CSF treatment in terms of a reduction in infectious complications, duration of antibiotic treatment or duration of hospitalization (77, 78, 83-85), larger randomized studies are needed to fully assess the value of rhGM-CSF in this setting. In this respect an interim analysis of 176 patients entered in a large randomized study with rhGM-CSF after chemotherapy and chest irradiation for limited SCLC is of interest (86). A significant reduction in leukopenia and neutropenia, but a significant increase in thrombocytopenia, infections and febrile days in the rhGM-CSF treated patients was demonstrated. As a consequence, the randomization was discontinued. It seems unlikely that these results were related to the rhGM-CSF dose chosen (250 μg/m^2 sc twice daily for 14 days) or the short interval between the last day of chemotherapy and the first dose of rhGM-CSF (one day). Although other studies do not support this, one could speculate that the described effect could be related to defective neutrophil migration as described previously during rhGM-CSF treatment after autologous BMT (87). The radiotherapy administered concurrently with the chemotherapy may have been a factor of relevance in the observed

thrombocytopenia, as both chemotherapy and rhGM-CSF induce proliferation of hematological progenitors. Scheduling of chemotherapy, radiotherapy and hematopoietic growth factors is essential, as demonstrated by the enhanced myelotoxicity observed in patients treated concomitantly with chemotherapy and rhGM-CSF (88). Overall, the toxicity profile of rhGM-CSF appears to be more pronounced in comparison with rhG-CSF. However, this seems especially true for high rhGM-CSF dose levels, and not for the dosages (5-10 μg/kg/day) that have demonstrated to be effective in clinical trials.

III-D. RhGM-CSF and chemotherapy dose-intensification

III-D1. Without bone marrow transplantation

With the appearance of hematopoietic growth factors better circumstances have been created for chemotherapy dose-intensification. Patients (n=32) with urothelial tumors refractory to standard dose chemotherapy were able to receive methotrexate and escalated doses of vinblastine (by 50%), doxorubicin (by 30%) and cisplatin (by 40%), when followed by rhGM-CSF (120-500 μg/m^2/day), administered either sc or by continuous iv infusion (89). Forty percent of the patients responded to chemotherapy. Sc and continuous iv administered rhGM-CSF showed similar hematological effects, but the sc route was better tolerated, with a MTD of 250 μg/m^2/day. In patients (n=5) with SCLC an accelerated chemotherapeutic regimen consisting of three cycles of cyclophosphamide-doxorubicin-vincristine and three cycles of cisplatin-etoposide alternating every week, increased the dose-intensity 1.5 fold. If rhGM-CSF (5 or 10 μg/kg/day by 2-hour iv infusion) was administered (n=5) at the occurrence of leukopenia ($< 1 \times 10^9$/l), a twofold dose-intensity was achieved (90). However, the significance of this study is limited as the group of patients was small and the way of rhGM-CSF administration is inferior to either continuous iv infusion or sc injection. Furthermore, an earlier start of rhGM-CSF might have been preferable. In chemotherapy-naive ovarian cancer patients (n=8), Rusthoven et al. were able to administer three or four three-weekly courses of cyclophosphamide (600 mg/m^2) and carboplatin (500 mg/m^2) on day 1 on an outpatient basis if followed by rhGM-CSF (10 μg/kg/day daily sc) on days 2-11 (91). In consecutive cycles prolonged neutropenia became dose-limiting at 600 mg/m^2 carboplatin and 600 mg/m^2 cyclophosphamide. Carboplatin repeatedly administered to seven patients at a dose of 600 μg/m^2 induced dose-limiting progressive thrombocytopenia ($< 25 \times 10^9$/l). The cyclophophamide/carboplatin regimen combined with rhGM-CSF resulted in a 2.1 fold relative increase in dose-intensity compared to a standard regimen. However, the authors considered this increase insufficient to warrant a phase III evaluation. Edmonson et al. were able to administer cyclophosphamide (1 g/m^2) and carboplatin (600 mg/m^2) every four weeks, when combined with rhGM-CSF given sc at a dose of 10 μg/kg every 12 hours for 14 days (92). In patients treated with intraperitoneal VP-16 (400 mg/m^2) and carboplatin, rhGM-CSF (500 μg/m^2/day) allowed carboplatin dose-escalation up to 600 μg/m^2 (93).

Of interest, it has recently been postulated that rhGM-CSF may replace autologous bone marrow rescue in selected cases (94). RhGM-CSF (250 $\mu g/m^2$/day), applied by continuous iv infusion after an intensive chemotherapy (BEAM) regimen, induced a full hematological reconstitution in three out of five patients with resistant non-Hodgkin's lymphoma not eligible for autologous BMT due to bone marrow involvement. A neutrophil count $> 0.5 \times 10^9$/l in these three patients was reached after a median of 26 days. A platelet count of $> 50 \times 10^9$/l was reached after a median of 35 days.

Other studies in which rhGM-CSF was used to attempt chemotherapy dose-intensification include patients with SCLC (95, 96), advanced urothelial tract tumors (97), metastatic gastro-intestinal cancer (98) and germ cell cancer (99). However, attempts to limit myelosuppression may not always be successful (100, 101).

These studies suggest that rhGM-CSF may allow chemotherapy dose-intensification. However apart from the dose, results may be influenced by the route of administration (102, 103) or time scheduling of rhGM-CSF (95, 104, 105). Although there is some evidence that rhGM-CSF may effect the platelet counts, as for rhG-CSF, chemotherapy dose-intensification may be limited by the degree of thrombocytopenia. Moreover, non-hematological toxicity will obtain a more prominent place. Certainly, further studies are needed to define the optimal treatment schedule with rhGM-CSF, which may differ for each chemotherapeutic regimen.

III-D2. With autologous bone marrow transplantation

In 1988 the first studies with rhGM-CSF after high-dose chemotherapy and autologous BMT in humans were published. Brandt et al. administered rhGM-CSF (2-32 μg/kg/day) by continuous iv infusion to 13 patients with breast cancer and 6 patients with melanoma (106). RhGM-CSF was given for 14 days starting the day after marrow reinfusion. Results were compared with a historical control group of 24 patients matched for age, diagnosis and treatment. The leukocyte and neutrophil recovery was accelerated in a dose-dependent way with maximum counts on the last day of rhGM-CSF administration. A neutrophil recovery $> 0.5 \times 10^9$/l was reached after 16 ± 6 days in the rhGM-CSF treated patients in comparison with 19 ± 5 days in the control group (NS). No effect on the platelet recovery was noted. Bacteremia was confirmed in 16% of the rhGM-CSF treated patients and 35% of the control patients. RhGM-CSF-related toxicity up to 16 μg/kg/day, comprised transient rash, oedema and myalgia, was mild. At 32 μg/kg dose-limiting side effects, consisting of generalized edema, weight gain, pleural and pericardial effusion, hypotension or myalgia were encountered. Nemunaitis et al. studied the effects of rhGM-CSF after autologous or syngeneic BMT in a phase I/II trial in 15 patients with lymphoid malignancies (107). RhGM-CSF (15-240 $\mu g/m^2$/day) was administered once daily by a 2-hour iv infusion for 14 days, starting directly after marrow reinfusion. Results were compared with a historical control group of 86 patients. At rhGM-CSF doses < 60 $\mu g/m^2$/day none of the patients achieved a neutrophil count $> 0.5 \times 10^9$/l by day 14, whereas five out of eight patients at doses ≥ 60 $\mu g/m^2$/day did. As only 5% of the patients in

the control group achieved the same, rhGM-CSF at doses $\geq$ 60 $\mu g/m^2$ appeared to accelerate the neutrophil recovery. There was no clear dose-response effect at doses $\geq$ 60 $\mu g/m^2$. However, the cohorts of patients per dose step were small (n=3). Two out of nine (22%) patients treated with $\geq$ 60 $\mu g/m^2$ had positive blood cultures before reaching a neutrophil count $>$ 0.5 $x10^9/l$ compared to 30% of the control patients. The average number of days with a temperature $>$ 38 °C (6$\pm$3 versus 12$\pm$7) as well as the mean time to platelet transfusion independence (29$\pm$5 versus 38$\pm$20) seemed to be shortened in patients receiving $\geq$ 60 $\mu g/m^2$ rhGM-CSF compared to the control group. The number of days in the hospital appeared to be reduced also by rhGM-CSF at these doses (29$\pm$9 versus 41$\pm$25).

In 16 patients with relapsed non-Hodgkin's lymphoma, undergoing autologous BMT, rhGM-CSF (11 $\mu g/kg/day$ by 4-hour iv infusion) accelerated the neutrophil recovery $>$ 0.5 $x10^9/l$ by a median of 6 days compared to a historical control group (108). No effect on the platelet recovery was observed. All patients developed neutropenic fever requiring iv antibiotic treatment. Toxicity of rhGM-CSF at this dose was mild. In 31 patients with resistant Hodgkin's disease receiving autologous BMT with (n=12) or without (n=19) subsequent rhGM-CSF (100-400 $\mu g/m^2/day$) by continuous iv infusion, a neutrophil count $\geq$ 0.5 $x10^9/l$ was reached 7 days earlier in the rhGM-CSF treated patients (109). Despite the faster neutrophil recovery, the infection rate, number of febrile days and the duration of hospitalization was similar for both groups.

The data from the first large multicentre randomized, placebo-controlled study with rhGM-CSF after autologous BMT for lymphoid cancer clearly demonstrated the effect of rhGM-CSF in that setting (110). Sixty-five patients received rhGM-CSF (250 $\mu g/m^2/day$) and 63 patients placebo by a 2-hour iv infusion for 21 days, starting within four hours after bone marrow reinfusion. A neutrophil count $>$ 0.5 $x10^9/l$ was reached seven days earlier in the rhGM-CSF group than in the placebo group (19 vs 26 days, $p<0.001$). Although the incidence of documented infections was not significantly different between the rhGM-CSF group (17%) and the placebo group (30%), the median duration of iv antibiotic treatment was shorter in the rhGM-CSF group (24 vs 27 days, $p=0.009$). The median duration of hospitalization was also shorter in the rhGM-CSF group (27 vs 33 days, $p=0.01$). Patients receiving rhGM-CSF required a median of 8 units of red blood cells, as compared with 10 units for patients receiving placebo ($p=0.047$). The platelet recovery was not significantly accelerated by rhGM-CSF. No toxic effects specifically ascribed to rhGM-CSF were encountered.

Preliminary data from two studies with rhGM-CSF after autologous BMT for non-hematological malignancies also demonstrated a beneficial effect. RhGM-CSF at a dose of 16 $\mu g/kg/day$ (continuous iv) reduced the duration of leukopenia $<$ 1.0 $x10^9/l$ by eight days in patients with neuroblastoma compared to controls ($p<0.005$) (111). This resulted in a shorter duration of antibiotic treatment and hospitalization. In patients with refractory germ cell cancer treated with rhGM-CSF (250 $\mu g/m^2/day$ continuous iv) after BMT, a reduction in neutropenia duration $<$ 0.5 $x10^9/l$ (12 vs 20 days in controls) and an earlier discharge from the hospital

(15 vs 24 days) was observed (112). In patients with neutropenic fever after autologous BMT, seven days of rhGM-CSF (2.8 μg/kg/day) treatment proved to be too short to significantly enhance neutrophil recovery (80). Fully published papers on a possible effect of rhGM-CSF on the survival times of patients are not yet available.

III-D3. With purged bone marrow reinfusion

Blazar et al. treated 25 patients with acute lymphoblastic leukemia with rhGM-CSF following reinfusion of 4-hydroperoxycyclophosphamide (4-HC) and anti-T or anti-B-cell lineage-specific antibody-purged autologous bone marrow (113). RhGM-CSF (16-256 $\mu g/m^2$/day) was given by either a 1-hour iv infusion four times daily or one 2-hour iv infusion. A historical control group consisted of 27 patients. The data obtained from this study suggested that in vitro purging of autologous bone marrow might reduce the responsiveness to rhGM-CSF administered in vivo after reinfusion. Five out of 16 patients receiving $\geq$ 60 $\mu g/m^2$/day rhGM-CSF for at least eight days reached a neutrophil count $>$ $1x10^9$/l by day 21 while 11 patients did not. In these five patients the higher neutrophil count was probably due to rhGM-CSF, as cessation of treatment induced a transient decrease in the neutrophil count by $>$ 50% within 48-72 hours. The response to rhGM-CSF was related to the number of CFU-GM progenitor cells infused per kg. The five responders had infused a median of 17.5 $x10^3$/kg CFU-GM, compared to 2 $x10^3$/kg in the non-responders ($p<0.001$). Nemunaitis et al. also found no response to rhGM-CSF (60-1000 $\mu g/m^2$/day) in seven patients receiving autologous bone marrow purged with 4-HC or etoposide, while 12 of the 15 patients receiving unpurged (n=9) or monoclonal antibody purged bone marrow (n=6) responded (114). However, in patients with lymphoid malignancies receiving mafosfamide-purged autologous bone marrow (n=5) followed by rhGM-CSF (10 μg/kg/day), the neutrophil recovery was not significantly different from patients receiving unpurged marrow followed by rhGM-CSF (115). The response to rhGM-CSF may depend on the number of myeloid progenitors administered to the patients.

III-D4. With allogeneic bone marrow transplantation

Data from studies with rhGM-CSF following allogeneic BMT are limited. A theoretical concern about the use of rhGM-CSF is the possibility of increasing the severity of graft-versus-host disease (GVHD) and the incidence of immunological graft rejection, as rhGM-CSF induces production of Interleukin-1 and tumor necrosis factor in monocytes (116-118), either of which may affect the severity of GVHD. In addition, rhGM-CSF increased the concentration of soluble IL-2 receptors in lymphoma, suggesting an activation of lymphocytes (119). However, no evidence has been obtained that rhGM-CSF influences the GVHD. A study with rhGM-CSF (60-1000 $\mu g/m^2$/day for 14 or 21 days) administered by a 2-hour iv infusion to, amongst others, 15 patients with marrow graft failure after allogeneic BMT, demonstrated no exacerbation of GVHD (114).

In the first randomized double-blind trial, 20 patients with leukemia received rhGM-CSF

(8 μg/kg/day continuous iv) or placebo for 14 days, starting the day of allogeneic marrow infusion (120). Ten patients (3 rhGM-CSF, 7 placebo) received busulphan and cyclophosphamide as an ablative regimen, 30 patients received melphalan or cyclophosphamide followed by TBI. Although a difference in neutrophil recovery between both arms was observed (day 14 rhGM-CSF group: 1.9 $x10^9$/l, placebo group 0.46 $x10^9$/l, $p<0.0001$), the duration of antibiotic treatment was longer in the rhGM-CSF group, whereas the incidence of documented bacterial infections was similar for both groups. No increased incidence of GVHD was reported in the rhGM-CSF group. Similar results were recently reported by Nemunaitis et al. (121). De Witte et al. observed higher neutrophil counts during the first six to ten days following allogeneic BMT in patients treated with rhGM-CSF (8 μg/kg/day) (n=29) compared to placebo-treated patients (n=28) (122).

Larger randomized studies are needed to reach conclusions on the role of rhGM-CSF after allogeneic BMT. No data are available concerning the effect of rhGM-CSF on survival after allogeneic BMT. The data that demonstrate that rhGM-CSF did not induce GVHD (121).

A further role of rhGM-CSF may lie in reversal of graft failure after BMT. Two studies demonstrated an increase in the neutrophil count during rhGM-CSF treatment in several patients with graft failure after allogeneic, autologous or syngeneic BMT (114, 123).

Several studies provide evidence that rhGM-CSF can be of clinical significance after autologous BMT. A dose of approximately 10 μg/kg/day seems to be an adequate and well tolerated dose to reduce the duration of neutropenia. However, very large studies may be needed to prove that rhGM-CSF treatment reduces the incidence of infections and the duration of antibiotic treatment and hospitalization. Moreover, an earlier discharge from the hospital is also related to the duration of severe thrombocytopenia, and rhGM-CSF has not clearly demonstrated to be effective in accelerating platelet recovery. Therefore, a combination with a hematopoietic growth factor enhancing thrombocytopoiesis (IL-3, IL-6) could be of additional value. In addition, the effect of rhGM-CSF on survival rates has yet to be established.

IV. Interleukin-3

IV-A. Biology

Murine Interleukin-3 (IL-3) was first purified from murine WEHI-3B myelomonocytic leukemic cells-conditioned medium in 1982 (124). Identification of the human gene encoding for IL-3 (or multi-CSF) by functional analysis of the products of a complementary DNA library followed (125, 126). The IL-3 gene is located on the long arm of chromosome 5 (127). In vivo, IL-3 is produced by mitogen- or antigen-stimulated T-lymphocytes, natural killer cells, mast cells and eosinophils (128-131). In vitro, it promotes the survival, proliferation and differentiation of multipotent hematopoietic stem cells and of the committed progenitor cells of the megakaryocyte, granulocyte/macrophage, erythroid, eosinophil, basophil and mast cell

lineages (132-135). IL-3 acts as a functional regulator of mature eosinophils and monocytes (4, 62, 136), but does not affect the function of mature neutrophils (137). In comparison with other CSF's, IL-3 has demonstrated to be a more potent stimulator of megakaryopoiesis in vitro (138-140). IL-3 has been expressed in various systems, including mammalian cells, yeast, B.Licheniformis and E.Coli. The rhIL-3 protein is glycosylated at various degrees, resulting in a broad range of molecular weights from 15-30 kD.

IV-B. Animal studies

Preclinical studies with homologous recombinant IL-3 in murine and primate models demonstrated a dose-dependent effect on the myelopoiesis, megakaryopoiesis and erythropoiesis (141-144). Continuous iv infusion of rhIL-3 (5-50 μg/kg/day for seven days) to primates induced a delayed and relatively modest leucocytosis, mainly caused by increases in lymphocyte, eosinophil and basophil counts, as well as an increase in reticulocyte and platelet counts (145). A dose of 20 μg/kg/day was needed to induce a consistent elevation of the different cell counts. RhIL-3 administered sc at doses of 11, 33 or 100 μg/kg/day for 14 days to rhesus monkeys resulted in a twofold to threefold increase in leukocyte counts, primarily caused by a dose-dependent elevation of basophils and eosinophils (146). No significant increase in neutrophils, red blood cells and platelets was noted. RhIL-3 administered sc or iv to cynomolgous primates at a dose of 20 μg/kg/day for 14 days following cyclophosphamide or 5-fluorouracil resulted in a higher neutrophil nadir count and a substantial shortening of the period of severe neutropenia ($< 0.5 \times 10^9$/l) compared to controls (147). In the rhIL-3 treated primates the myeloid recovery was marked by an eosinophilia and basophilia, not observed in the control animals. The erythroid recovery was not accelerated by rhIL-3. Although the platelet recovery appeared to occur earlier with rhIL-3, the interpretation remained difficult due to large variations in platelet recovery in the control animals. All rhIL-3 treated primates developed localized facial and extremity swelling 8-11 days after start of rhIL-3 treatment. In addition, a pruritic rash was noted. Biopsies revealed perivascular infiltrates of neutrophils and eosinophils. These symptoms disappeared within one week after rhIL-3 discontinuation. In all rhIL-3 treated animals a mild splenomegaly was observed. However, the studies with non-homologous rIL-3 in primates suffered from considerable lack of specificity and subsequently were not representative for the full potential activity of rIL-3 (141, 148).

IV-C. RhIL-3 and standard-dose chemotherapy

In the first phase I/II studies in humans without cytostatic treatment, rhIL-3 (30-500 $\mu g/m^2$/day) demonstrated a multilineage stimulation of the hematopoiesis (149-152). A similar response was induced by a 4-hour iv infusion of rhIL-3 (30-1000 $\mu g/m^2$/day for 28 days) (153). In these studies, a pronounced increase in leukocyte and platelet counts was noticeable from the second week of treatment onwards. During sc treatment, increases in neutrophils were rhIL-3 dose-dependent, while 250 $\mu g/m^2$/day rhIL-3 appeared to be at least equally effective as 500 $\mu g/m^2$

in enhancing the platelet count. No clear dose-response effect could be demonstrated for the 4-hour iv infusion. As expected, the magnitude of the rhIL-3 effect on the leukocyte and platelet counts appeared to be related to the remaining bone marrow function.

RhIL-3 up to 250 $\mu g/m^2$/day administered sc or by 4-hour iv infusion was in general reasonably well tolerated (149-153). Frequently observed side effects were fever and headache, sometimes accompanied by neck rigidity. Less frequently encountered side effects were chills, flushing, bone pain, rash, nausea, vomiting and peripheral oedema. At 500 $\mu g/m^2$/day rhIL-3, administered either sc or as a 4-hour iv infusion dose-limiting toxicity was encountered, consisting of severe headache accompanied by neck rigidity (149, 151, 152), bone pain (152), decrease in platelet counts (152), severe headache combined with nausea and vomiting and recurrent urticaria (153). RhIL-3 administered as an iv bolus at 125 $\mu g/m^2$ caused transient acrocyanosis and chills in all three patients (152).

These data suggest that a sc dose of 250 $\mu g/m^2$/day could be the desirable dose to be used in further trials.

The first data from trials in which rhIL-3 was used to reduce chemotherapy-induced myelotoxicity have become available only recently. We treated 20 patients with advanced ovarian cancer with E.Coli-derived rhIL-3 (1-15 $\mu g/kg/day$) following every other course of carboplatin and cyclophosphamide (154). RhIL-3 was administered sc or continuously iv for seven days, starting four days after chemotherapy. The cycles without rhIL-3 served as control cycles. At 10 $\mu g/kg/day$ both the neutrophil and platelet nadir were higher than in the control cycles. In addition, the hematological recovery was accelerated by rhIL-3, leading to less frequent chemotherapy postponement (6% of the rhIL-3 cycles versus 46% of the control cycles, $p<0.01$). The toxicity profile and incidence of the side effects were very similar to those reported in the early phase I/II trials (149-151). The MTD in this study was 10 $\mu g/kg/day$, as headache became dose-limiting at 15 $\mu g/kg/day$ rhIL-3. In another study 15 relapsed SCLC patients were treated with (B.Licheniformis-derived) rhIL-3 (1-16 $\mu g/kg/day$ sc) following chemotherapy (155). The compiled data from 8 and 16 $\mu g/kg$ demonstrated that the recovery of leukocytes, neutrophils and platelets was accelerated compared to a previous control cycle. The side effects of rhIL-3 resembled those of the previous study and may be related to interleukin-6 release induced by rhIL-3. In addition, rhIL-3 augmented tumor necrosis factor alpha plasma levels.

Demetri et al. administered rhIL-3 (0.15-5 $\mu g/kg/day$) sc to 16 chemotherapy-naive patients with advanced sarcomas prior and after chemotherapy (156). In the pre-chemotherapy phase rhIL-3 induced a modest dose-dependent stimulation of the hematopoiesis. At 2.5 and 5 $\mu g/kg$ the neutrophils increased an average of 20% by day 10. Platelets demonstrated a more delayed response with an increase of 40% by day 18. In the post-chemotherapy phase rhIL-3, administered for 11 days at doses < 2.5 $\mu g/kg/day$, did not demonstrate a significant effect on the neutrophil and platelet recovery in comparison with a previous control cycle. Data from the 2.5 and 5 $\mu g/kg$ dose step were not yet available. RhIL-3 up to doses of 2.5 $\mu g/kg/day$

administered sc to NSCLC patients on chemotherapy treatment (n=12) also demonstrated no effect on the neutrophil and platelet counts (157). Although 5 μg/kg rhIL-3 did not increase the neutrophil and platelet nadir counts, it appeared to accelerate the recovery from the nadir counts. However, the number of days with neutropenia (< 0.5 $x10^9$/l) or thrombocytopenia (< 50 $x10^9$/l) were not reduced. No clear effect on the reticulocyte counts could be demonstrated. Data from a multicentre, randomized double-blind placebo-controlled study with rhIL-3 administered continuously iv to patients with relapsed malignant lymphoma also suggest that a dose of 5-10 μg/kg/day rhIL-3 is an effective dose for accelerating the neutrophil and platelet recovery after chemotherapy (158).

These early phase I/II studies demonstrated that a dose of 5-10 μg/kg/day rhIL-3 may affect the duration of chemotherapy-induced neutropenia and thrombocytopenia. However, the effect on the neutrophil and the platelet nadir counts seems limited, as could be expected from the early phase I/II trials demonstrating a relatively late effect on the leukocyte and platelet counts. Therefore, depending on the chemotherapy schedule, rhIL-3 may be of greater value in reducing the duration of myelosuppression, rather than in elevating the nadir counts. Of the hematopoietic growth factors that have been used in clinical trials, rhIL-3 seems to have the most consistent effect on platelet counts.

Studies in which rhIL-3 is administered to febrile neutropenic patients in order to accelerate (hematological) recovery have not yet been reported. In view of the delayed hematological effects, it is questionable whether such attempts would be successful.

Side effects of rhIL-3 after chemotherapy in the effective dose range were frequent, but generally tolerable. Both the toxicity profile and the incidence of side effects were very similar to those in the early phase I/II studies (149-153).

IV-D. RhIL-3 and bone marrow transplantation

The first preliminary data from studies with rhIL-3 after BMT became available in 1991. Fibbe et al. treated 14 evaluable patients in second or subsequent remission from malignant lymphomas with rhIL-3 by continuous infusion for 14 days starting the day after autologous bone marrow reinfusion (159). The rhIL-3 dose administered ranged from 0.25 to 15 μg/kg/day. Five eligible patients were not treated with rhIL-3 and served as a control group. A neutrophil count > 0.5 $x10^9$/l was reached after a median time of 17 days in patients treated with 10 μg/kg/day versus 28 days in control patients and patients receiving 0.25 μg/kg/day. A platelet count > 50 $x10^9$/l was reached after 19 days at 10 μg/kg versus 30 days in patients receiving no rhIL-3 or 0.25 μg/kg. A recovery to 1% reticulocytes was shortened by 19 days at 10 μg/kg. A dose of 5 μg/kg showed intermediate effects. At 15 μg/kg dose-limiting side effects were encountered in four out of five patients.

In patients with metastatic breast cancer (n=11) receiving high-dose chemotherapy and purged autologous bone marrow, rhIL-3 was given by continuous iv infusion starting the day of marrow reinfusion (160). RhIL-3 at 50 and 250 μg/m^2/day accelerated the neutrophil

recovery > 0.5 x10^9/l by a median of 8 days compared to a historical control group. At 500 $\mu g/m^2$/day dose-limiting side effects were encountered in both patients treated at that dose step, consisting of fluid retention in both patients and a Parkinson-like syndrome in one patient. In this study the effect of 250 $\mu g/m^2$/day was not superior to 50 $\mu g/m^2$.

Although the data are limited and preliminary, these studies seem to demonstrate that rhIL-3 can accelerate the hematological recovery after BMT. As the MTD seems to have been established (10 μg/kg/day), further studies at this dose are warranted to define the actual value of rhIL-3 in BMT patients. In view of its delayed hematological effects, a combination with other growth factors may be more effective. Data concerning the efficacy of rhIL-3 in patients with graft failure are not yet available.

V. Hematopoietic growth factors and peripheral stem cell harvesting

Although the administration of hematopoietic growth factors can enhance the hematological recovery after BMT, further improvement might be achieved by the addition of peripheral harvested stem cells. Dührsen et al. noted increases in all types of maturational stages of circulating progenitor cells during and until two days after rhG-CSF administration (0.3-60 μg/kg/day) in cancer patients (161). This effect was rhG-CSF dose-dependent. Trials in which rhG-CSF is administered after a standard dose chemotherapy in order to increase the number of peripheral stem cells for harvesting have recently been initiated (162-164). Bolwel et al. reinfused autologous bone marrow and peripheral stem cells harvested after chemotherapy and rhG-CSF (5 μg/kg/day) in 13 patients with non-Hodgkin's lymphoma or Hodgkin's disease (163). RhG-CSF was restarted at 16 μg/kg/day on the day of peripheral stem cell reinfusion. A neutrophil count > 0.5 x10^9/l was reached nine days after BMT (10 days after peripheral stem cell reinfusion) compared to 20 days in a historical control group after BMT only. A platelet count > 20 x10^9/l was reached in 13 and 21 days after BMT in the study group and the control group, respectively. As a consequence, the duration of hospitalization was reduced by nine days. Socinski et al. demonstrated that chemotherapy followed by rhGM-CSF (4-32 μg/kg/day) resulted in a synergistic effect on the peripheral CFU-GM release as the CFU-GM numbers increased by approximately 60-fold compared to the pretreatment baseline (165). Siena et al. demonstrated a fivefold increase in circulating $CD34^+$ (a surface antigen considered to be characteristic for undifferentiated hematopoietic progenitor cells) mononuclear cells after cyclophosphamide (7 g/m^2) followed by rhGM-CSF (5.5 μg/kg/day continuous iv for 14 days) (166). $CD34^+$ marrow cells autotransplanted in lethally irradiated baboons completely restored haematopoiesis in vivo (167).

Subsequently, Gianni et al. observed an up to 1000-fold increase in peripheral CFU-GM after cyclophosphamide (7 g/m^2) followed by rhGM-CSF (5.5 μg/kg/day continuous iv) treatment (168). These circulating progenitor cells were harvested by leukapheresis and used

in the setting of autologous BMT. The results demonstrated a significant faster recovery of neutrophils and platelets. In addition, mucositis, although present in all patients, was less severe in patients transplanted with rhGM-CSF-exposed progenitor cells compared to the control group. Other studies also showed increased yields of harvested peripheral progenitor cells after chemotherapy and rhGM-CSF compared to chemotherapy only (169, 170).

Although the theoretical concern was raised that rhGM-CSF might induce a differentiative shift of pluripotent stem cells towards lineage-restricted progenitors (165), several studies have demonstrated that reinfusion of autologous progenitor cells, harvested from the peripheral blood after rhGM-CSF priming, can fully restore hematopoiesis (170-173).

These studies demonstrate that rhG-CSF and rhGM-CSF, especially in combination with a single course of chemotherapy, can increase the number of circulating hematological progenitor cells, resulting in higher yields during harvesting by leukaphoresis. Reinfusion of these progenitor cells in combination with bone marrow progenitor cells can accelerate the recovery of the neutrophils and platelets compared to reinfusion of bone marrow progenitors alone. Recent data also suggest a potential role of rhIL-3 in enhancing peripheral stem cell harvest (174).

At this stage the combined infusion of bone marrow cells and peripheral harvested stem cells provide the fastest bone marrow restoration after ablative chemotherapy. The role of hematopoietic growth factors after reinfusion of peripheral harvested progenitors has yet to be studied, as this combination may provide an opportunity to restore hematopoiesis without bone marrow reinfusion.

VI. Erythropoietin

VI-A. Biology

Erythropoietin (Epo) was the first hematopoietic growth factor to be identified. Human urinary Epo was purified in 1977 from patients with aplastic anemia (175). Subsequently, Epo was cloned and expressed in mammalian cells (176, 177). The MW of this glycoprotein is 34-39 kD (175). The gene encoding for Epo is located on the long arm of chromosome 7 (178). Epo is mainly produced in the peritubular interstitial cells of the kidney (179, 180) and to a small extent in the liver and bone marrow macrophages (181, 182). Epo is the main regulator of red blood cell production by promoting proliferation and differentiation (183, 184) and preventing apoptosis (185) of erythroid progenitors. In addition, Epo modulates the proliferation of megakaryocytes in the presence of an additional growth factor (186-188).

VI-B. Studies involving chemotherapy-treated animals

In a study conducted by Matsumoto et al., rats were treated with either 5-FU (150 mg/kg) or cisplatin (8 mg/kg) by an iv bolus injection on day 0 (189). In both groups rats became anemic.

In the 5-FU-treated rats rhEpo, administered on alternating days from days 0-12 by single iv bolus injection, hastened the recovery of the Hb levels at doses of 1700-3400 IU/kg. In the cisplatin-treated group with less pronounced anemia rhEpo, given at doses of 21-170 IU/kg on alternating days from days 13-31, significantly increased the Hb levels in a dose-dependent fashion. Of interest, in contrast to other GF's (35, 88), simultaneous administration of rhEpo and chemotherapy did not enhance myelotoxicity. In fact, a more efficient anemia-reducing effect was reached by rhEpo treatment on alternating days from day eight before 5-FU injection to day 12 after 5-FU injection as the Hb value did not fall below normal levels at rhEpo doses of 1700-3400 IU/kg.

VI-C. RhEpo and standard dose chemotherapy

The anemia of cancer patients may, to a smaller or larger extent, be related to inadequate Epo production (190). Miller et al. demonstrated that the blunted Epo response in anemia of cancer patients was worsened by chemotherapy. However, they found no difference in endogenous Epo response between anemic patients treated with a cisplatin-containing regimen and patients treated with a regimen without cisplatin.

RhEpo administration has demonstrated ability to ameliorate anemia associated with renal failure (191), rheumatoid arthritis (192), multiple myeloma (193) and zidovudine treatment (194). Also cancer patients with anemia due to bone marrow involvement demonstrated an increase in hemoglobin (Hb), hematocrit (Ht) and red blood cell (RBC) count during four weeks of rhEpo administration twice weekly (iv bolus) at doses of 300 and 450 IU/kg (195). At these doses no rhEpo-related side effects were observed. RhEpo has also shown to be of value in correcting chronic anemia in cancer patients (196-198).

In a phase I/II study 30 patients with chemotherapy-induced anemia (Hb $<$ 11 g/dL) were treated with rhEpo at doses of 25-300 IU/kg given during five consecutive days in each of four weeks (199). RhEpo was administered by a 5-min iv infusion, starting concomitantly with a chemotherapy course not containing cisplatin. RhEpo demonstrated a dose-dependent Hb increase ($>$ 10%), with 11 out of 13 patients at 200 and 300 IU/kg responding compared to four out of 17 patients at 25-100 IU/kg. At the two highest dose steps responses occurred earlier, with five patients responding after one and two weeks, respectively. At doses $\leq$ 100 IU/kg the response usually occurred in the last week of treatment. Patients responding to rhEpo demonstrated at the end of their previous chemotherapy cycle a decrease in the Hb value. Thus, rhEpo did not only prevent worsening of the anemia but also induced a significant improvement of HB levels. Side effects of rhEpo were scarce.

In an open, three arm, randomized trial, groups of 12 patients with SCLC received no rhEpo, or 150 or 300 IU/kg rhEpo thrice weekly by sc injection following each of a maximum of 6 chemotherapy cycles (200). An interim analysis demonstrated a fall in Hb levels in the control group after the first chemotherapy course. A similar fall was delayed until after the second and third course in patients treated with 150 and 300 IU/kg rhEpo, respectively. More

RBC units were transfused in the control group compared to rhEpo treated patients. A similar analysis of platelet transfusions demonstrated no significant differences. Preliminary data from a randomized trial with rhEpo (300 IU/kg sc biweekly) after adriamycin-containing chemotherapy for breast cancer showed a (not significant) trend towards higher average and absolute Hb values in the rhEpo group (201). In a series of double-blind placebo-controlled trials, patients treated with chemotherapy were randomized to rhEpo (150 IU/kg) or placebo treatment three times per week (196). Patients were divided into two groups depending on the presence (n=157) or absence (n=132) of cisplatin in the regimen. Both rhEpo-treated groups demonstrated an increase in Ht values compared to their controls. When both groups were combined, the rhEpo-treated patients had lower RBC transfusion requirements during months two and three (1.04 units versus 1.81 units in controls), but not during the first month, suggesting a lag phase of one month in rhEpo efficacy. Preliminary data from a small study (n=10) with rhEpo (50 IU/kg sc thrice weekly) showed an amelioration of cisplatin-related anemia (202).

VI-D. RhEpo and bone marrow transplantation

Studies with rhEpo after BMT have recently been initiated. Link et al. administered rhEpo (150 IU/kg/day by continuous iv infusion) to 19 patients after allogeneic BMT (203). Results were compared with a historical control group of 43 patients. A Ht of 0.35 was reached after a median of 22.5 days (range 16-24) in the rhEpo group versus 32 days (range 14-187) in the control group. RBC transfusion independency was reached after a median of 16 days (range 10-54) and 24.5 days (11-79) in the rhEpo and control group, respectively. In addition, they observed an acceleration of the reticulocyte increase in the rhEpo-treated group. No side effects of rhEpo administration were noted. RhEpo administration (150 and 300 IU/kg twice weekly sc) for three weeks also increased the Hb levels in five patients with anemia (Hb $<$ 8 g/dl) beyond 3 weeks post-BMT (204). No adverse effects of rhEpo were observed. Preliminary data from a study with rhEpo (200 IU/kg/day as an iv bolus) administered from the day after autologous BMT for solid tumors, suggested a trend towards earlier engraftment and the need for fewer RBC transfusions in patients receiving rhEpo (n=6) (205).

As data from studies with rhEpo after chemotherapy are limited, its precise value is still difficult to assess. Several studies demonstrate a delayed effect on the erythropoiesis suggesting that a prolonged period of administration may be required. During standard dose chemotherapy regimens this may present difficulties as simultaneous administration of GF's and chemotherapy may enhance myelotoxicity. However, several of the previous mentioned studies do not support this concern for rhEpo. Only cancer patients in frequent need of RBC transfusions may benefit from rhEpo administration. In addition, a possible reduction of the risk of transfusion-related infectious complications could be considered as an advantage of rhEpo over RBC transfusions. However, the costs related to rhEpo administration are high and may exceed the costs related to RBC transfusions. Certainly, in most chemotherapy regimens physicians will be more

concerned with neutropenia and thrombocytopenia. Consequently, the use of hematopoietic growth factors reducing this kind of myelotoxicity may be preferred.

VII. Macrophage colony-stimulating factor

VII-A. Biology

Human macrophage CSF (M-CSF) was purified from urine (206) and a pancreatic carcinoma cell line (207). In vivo the main source of M-CSF are fibroblasts (208), but its synthesis can also be induced in endothelial cells (209) and monocytes/macrophages (210-212) in response to other cytokines. M-CSF is a lineage-restricted CSF, stimulating proliferation and differentiation of cells of the monocyte/macrophage lineage in vitro (213). Furthermore, M-CSF potentiates functions of mature macrophages such as tumoricidal activity (214-217) and production of hematopoietic growth factors (218). The gene encoding for human M-CSF is located on the short arm of chromosome 1 (219). Three different cDNAs have been isolated (220-222) encoding for M-CSF α, ß and τ with MW ranging from 70 to 90 kD. Mammalian cells (221) and yeast (223) are the main sources of rhM-CSF.

VII-B. M-CSF and chemotherapy

The first clinical studies in humans were conducted with purified human M-CSF. Urinary M-CSF (8×10^6 U/day), administered for seven days by a 2-hour iv infusion, induced an increase in peripheral monocyte counts in six healthy volunteers (224). No effect on other blood cell counts was noted. In 33 patients with urogenital malignancies, urinary M-CSF administration (8×10^6 U/day by 2-hour iv infusion), starting the day after chemotherapy, increased leukocyte nadir counts, accelerated leukocyte recovery to 2×10^9/l, and increased neutrophil nadir counts compared to a previous control cycle (225). The duration of neutropenia was not shortened by urinary M-CSF. Severe M-CSF-related side effects were not observed. Patients with gynaecological malignancies and on chemotherapy treatment received urinary M-CSF (8×10^6 U/day for seven days), administered by a 2-hour iv infusion after the second chemotherapy course (224). The duration of neutropenia ($< 0.5 \times 10^9$/l) and thrombocytopenia ($< 100 \times 10^9$/l) was shorter in the M-CSF cycle. In addition, the platelet nadir was increased in that cycle.

Urinary M-CSF (8×10^6 U/day for 14 days) starting 1-3 days after BMT for myeloid leukemia ($n=45$), hastened the neutrophil recovery to 0.5×10^9/l by a mean of approximately four days compared to 52 control patients ($p < 0.05$) (226). Again urinary M-CSF demonstrated no severe side effects at this dose. In addition, the leukemic relapse rate was similar for the urinary M-CSF and control group.

Data of rhM-CSF after chemotherapy are not yet available. Phase I trials have demonstrated a dose-dependent increase in peripheral monocyte counts (227-230), whereas thrombocytopenia may become the dose-limiting factor (228, 230).

Until now, phase I data provide little or no evidence that rhM-CSF will ameliorate chemotherapy-induced myelosuppression, while purified urinary M-CSF will be available in only limited quantities.

In vitro data demonstrating M-CSF-enhanced tumoricidal activity of mature monocytes/macrophages (214-217) and M-CSF-enhanced macrophage antibody-dependent cellular cytotoxicity (231, 232) suggest that rhM-CSF may be of use as an antitumor drug. In addition, animal data suggest that rhM-CSF, in combination with e.g. a tumor-specific monoclonal antibody, may be of use in the treatment of patients with metastasized cancer (233). In phase I studies with rhM-CSF tumor response was observed in incidental cases of leiomyosarcomas (227).

VIII. Newer hematopoietic growth factors and other cytokines with growth factor properties

VIII-A. Interleukin-1

In vitro studies have shown that Interleukin-1 (IL-1) stimulates release of other hematopoietic growth factors (234-236) and affects early myeloid precursors (237, 238). RhIL-1 administered to mice stimulated generation of myeloid, megakaryocytic and early erythroid progenitor cells (239). RhIL-1 accelerated myeloid recovery in rodents and primates following chemotherapy (238, 240, 241) and irradiation (242). Furthermore, rhIL-1 administration in mice resulted in enhanced survival after potentially lethal irradiation and allogeneic BMT (243).

Recently, data from a phase I trial with rhIL-1ß administered for two days to 19 patients with gastrointestinal cancer before and after myelosuppressive doses of 5-fluorouracil became available (244). In the pre-chemotherapy phase, rhIL-1ß (0.002-0.1 μg/kg/day as a 30-min iv infusion) induced a dose-dependent increase in neutrophil counts, which occurred earlier at higher dose steps. The counts rose within two hours after infusion and returned to baseline levels within 48 hours. A modest and delayed thrombocytosis occurred a median of 14 days after rhIL-1ß infusion. A fairly similar hematological response was observed by Tewari et al. (245). Hypotension became the dose-limiting toxicity of iv rhIL-1ß at 0.1 μg/kg/day. Although rhIL-1ß (starting one day after chemotherapy) appeared to reduce the duration of chemotherapy-induced neutropenia, comparison with a previous control cycle demonstrated no significant difference. After chemotherapy, rhIL-1ß showed no effect on the platelet counts, the incidence of febrile neutropenia or mucositis. However, in view of the phase I character of this study this was not remarkable. In eight patients with advanced urologic cancers, the rhIL-1ß analogue OCT43 (50,000 U) was administered sc once or twice if chemotherapy-induced neutropenia ($< 0.5 \times 10^9$/l) occurred (246). OCT43 administration resulted in a significantly shorter duration of neutropenia ($< 1 \times 10^9$/l) compared to a previous control cycle (6 ± 1.5 days versus 8.9 ± 2 days in controls).

Following autologous BMT rhIL-1ß (0.01-0.02 μg/kg/day), given to nine patients as a 30-min iv infusion on days 0-4 after marrow reinfusion, accelerated the neutrophil recovery to 0.5 $x10^9$/l by a median of 11 days compared to a historical control group (247). In contrast, in another phase I trial, Vredenburgh et al. observed no effect of rhIL-1ß (0.004-0.032 μg/kg/day sc for seven days) on the hematopoietic recovery after ablative chemotherapy and autologous BMT for breast cancer (n=14) and melanoma (n=2) (248).

VIII-B. Interleukin-6

Interleukin-6 (IL-6) affects, in conjunction with other hematopoietic growth factors, the maturation of multipotential, myeloid, erythroid and megakaryocytic progenitors in vitro (56, 249-251). IL-6 also stimulates the proliferation of granulocyte-macrophage progenitors (252) and the maturation of megakaryocytes (253, 254). In vivo administration of rhIL-6 in mice and primates induced a dose-dependent increase in peripheral platelet counts (253, 255). In addition, rhIL-6 has the ability to accelerate platelet recovery after sublethal irradiation (256-258) or chemotherapy (259) in animals. Phase I studies with rhIL-6 in humans have recently been initiated.

VIII-C. Stem cell factor and Interleukin-11

As the search for new cytokines continues, other candidates for ameliorating chemotherapy-induced myelosuppression are bound to come forward. The stem cell factor (SCF, c-kit ligand) augments the proliferation of myeloid, lymphoid, erythroid and megakaryocytic progenitors, either directly or in conjunction with other hematopoietic growth factors (260-262). Preliminary data from animal studies demonstrated an enhanced hematopoietic recovery after TBI with or without BMT if recombinant stem cell factor was administered (263, 264).

Interleukin 11 (IL-11) directly supports the proliferation of granulocyte/macrophage colonies in vitro (265). In addition, it synergizes with IL-3 and IL-4 in supporting the proliferation of early murine hematopoietic progenitor cells by shortening the G_0 period (265, 266). Preliminary data from a study in mice treated with TBI followed by syngeneic BMT demonstrated an accelerated neutrophil and platelet recovery in animals given rhIL-11 (100 μg/kg/day) (267).

IX. The combined use of hematopoietic growth factors

In vitro, hematopoietic growth factors in many cases act additively or synergistically on the proliferation and differentiation of hematopoietic progenitor cells (55, 56, 249-251, 261, 266). In primates similar effects have been demonstrated (144, 145). Recently, studies in humans have been initiated to investigate whether the use of a combination of growth factors is more effective in enhancing hematopoiesis, especially thrombopoiesis, compared to the use of a

single agent.

In a phase I trial 14 cancer patients were treated with five days of rhIL-3 (30-500 $\mu g/m^2$/day sc) followed by rhGM-CSF administered for 10 days (250 $\mu g/m^2$/day sc) (268). The combination regimen demonstrated no synergistic effects on myelopoiesis or thrombopoiesis compared to the use of rhGM-CSF and rhIL-3 alone. However, the group of patients was very small and changes in the administration schedule and dose of the factors may provide better results. The combination of rhIL-3 followed by rhGM-CSF was also studied in patients on chemotherapy treatment (269, 270). Brugger et al. treated 36 cancer patients with chemotherapy followed by rhIL-3 (250 $\mu g/m^2$/day on days 1-5 sc) and rhGM-CSF (250 $\mu g/m^2$/day on days 6-15 sc) (269). Preliminary results were compared with patients given rhGM-CSF after chemotherapy and patients treated with chemotherapy only. The patients treated with a hematopoietic growth factor demonstrated a faster neutrophil recovery compared to patients not treated with a growth factor. There was no difference in neutrophil recovery between the rhIL-3 plus rhGM-CSF group and the rhGM-CSF group. In general, the platelet recovery was not hastened by the administration of either the combination of rhIL-3 plus rhGM-CSF or rhGM-CSF alone. This may have been related to the short duration of rhIL-3 administration in this study.

Another combination currently under investigation is the administration of rhG-CSF combined with rhGM-CSF after BMT (271). In addition, there is some preliminary evidence that sequential administration of rhGM-CSF and rhG-CSF may be effective in patients with delayed engraftment not responsive to single growth factor treatment (272).

A combination of growth factors may also facilitate the harvesting of peripheral stem cells. In vitro administration of rhIL-3 increased the number of hematopoietic progenitor cells harvested after in vivo stimulation with rhGM-CSF or rhG-CSF (273). Trials in which a combination of hematopoietic growth factors are used to enhance mobilization of progenitor cells for peripheral stem cell harvesting are in progress (271, 274).

At this stage it is unclear whether combining hematopoietic growth factors will have advantages over the use of a single growth factor. Certainly, studying combinations of growth factors seems worthwhile, especially since single growth factors (rhIL-3) have demonstrated a lag-phase in enhancing peripheral platelet counts. In this respect, the recent construction of a rhGM-CSF/rhIL-3 fusion protein, which in vitro demonstrated enhanced hematopoietic colony-stimulating activity compared to rhGM-CSF or rhIL-3 alone is of interest (275).

X. Hematopoietic growth factors and tumor cell proliferation

X-A. Non-hematopoietic tumors

Concerns have been raised regarding the induction of tumor proliferation by hematopoietic growth factors in vivo. Some in vitro studies have shown that hematopoietic growth factors

may enhance the in vitro proliferation of non-hematopoietic tumor cell lines (276-281) due to the presence of hematopoietic growth factor receptors on these tumor cells (278, 280, 282-284). However, the effects are not only supportive but can also be inhibitory (285-287). Moreover, the supportive effects of hematopoietic growth factors are noticed in only a minority of the cases (278, 288-290). Certainly the debate on positive or negative effects of in vivo administered growth factors on non-hematopoietic tumor growth will continue. At this stage there is no overwhelming evidence for a clinical significance of these in vitro effects. However, this may be due to the fact that probably very large randomized studies are required to demonstrate clinically a tumor cell proliferation induced by hematopoietic growth factors.

X-B. Myeloid leukemia

Similar concerns have been expressed for myeloid leukemia patients treated with hematopoietic growth factors, as these factors can stimulate proliferation of myeloid leukemia cells (291-296). Data from a large randomized trial with rhG-CSF after intensive induction chemotherapy in relapsed or refractory acute leukemia did not provide evidence for a negative effect of rhG-CSF as the rates of complete remission and relapse were similar for the rhG-CSF treated group and a control group (29). Other, albeit smaller studies, seem to support this observation (297, 298).

In order to take advantage of CSF-enhanced proliferation of leukemic cells, it was suggested that CSF's should be administered prior to chemotherapy to increase the percentage of leukemic cells in the S-phase (9). In vitro, CSF's can recruit kinetically quiescent AML cells into G_1-and S-phase, thus making them more sensitive to cell-cycle specific chemotherapeutic drugs (299, 300). Recently a similar in vivo recruitment of leukemic cells has been demonstrated for rhGM-CSF (301, 302), rhG-CSF (303), and rhIL-3 (304). In vitro data also suggest that hematopoietic growth factors may augment the cytotoxic effect of topoisomerase II-targeting drugs in leukemia. Human myeloid leukemia cells responsive to rhG-CSF demonstrated enhanced expression of DNA topoisomerase II in the presence of rhG-CSF (305). Although several studies demonstrated considerable remission rates in AML patients treated with CSF's prior, or during, chemotherapy (301-303), it should be noted that these were studies not dealing with survival rates. In this respect, data from a study in which 56 patients with de novo AML were treated with rhGM-CSF (125 $\mu g/m^2$/day sc) prior to and during ara-C and daurubicin are of interest (306). This treatment schedule was predictive ($p < 0.013$) of both a lower complete remission rate and survival probability for these patients compared to control patients ($n = 175$) receiving the same dose and schedule of ara-C without rhGM-CSF. Certainly, the scheduling of CSF's has to be optimized and large randomized studies are needed to study whether CSF's given prior or during chemotherapy in myeloid leukemia are able to increase response and survival rates.

XI. Conclusions

The introduction of rh hematopoietic growth factors to clinical oncology and hematology has created the opportunity to investigate whether, and to what degree, these cytokines can ameliorate chemotherapy-induced myelosuppression. For both rhG-CSF and rhGM-CSF there is evidence that these CSF's can reduce the duration of neutropenia following standard dose chemotherapy as well as ablative chemotherapy plus BMT. These results may be further improved by defining the optimal dose and administration schedule of these cytokines, especially during multiple cycles of standard dose chemotherapy. Unfortunately, it seems not unlikely that every chemotherapeutic regimen will need a different cytokine scheduling, depending on the pharmacokinetics of the cytotoxic drugs used. Promising as the results may be, it has yet to be clearly demonstrated that application of rhG-CSF or rhGM-CSF will lead to a decrease in the rate of infectious complications, antibiotic use and hospitalization, and subsequently to a decrease in health care costs for but a few therapeutic regimens. There is some evidence that the use of rhG-CSF after standard dose chemotherapy can reduce the costs of health care (307). This despite the fact that the price label attached to a prolonged use of rh hematopoietic growth factors is quite impressive. RhGM-CSF, rhG-CSF and rhEpo are currently the only hematopoietic growth factors commercially available for clinical use. However, at this stage rhGM-CSF is only available in a limited number of countries. At this stage, regulatory agencies, such as the Federal Bureau of Drug Administration, have approved the use of rhG-CSF in solid tumor chemotherapy, whereas rhGM-CSF has been approved for a BMT setting. In addition, rhG-CSF appears to have a more favorable toxicity profile compared to rhGM-CSF. However, this seems only true for high rhGM-CSF dose levels, and not for the dosages (5-10 μg/kg/day) that have demonstrated to be effective in clinical trials. Comparing the efficacy of the same hematopoietic growth factors from different sources is difficult given the wide variety in specific activity of the different proteins, depending on the industrial source. An, albeit minor, advantage of rhGM-CSF over rhG-CSF could be its effect on thrombopoiesis. However, this effect remains controversial, as the number of studies demonstrating an effect of rhGM-CSF on the platelet counts is at least equalled by the number of studies in which no effect on the platelet counts was observed.

At the moment, rhIL-3 seems the first candidate for reducing chemotherapy-induced thrombocytopenia. In addition, it also stimulates neutropoiesis. It may well be that the earlier acting hematopoietic growth factors have a less favorable toxicity profile as these factors act on several cell lineages and may enhance the release of multiple cytokines, partially responsible for the side effects. Other possible candidates for stimulating thrombopoiesis (IL-1, IL-6) have only reached the early stages of clinical investigation, and the same holds true for combinations of growth factors.

Although hematopoietic growth factors have demonstrated ability to reduce chemotherapy-induced myelosuppression it remains to be determined whether this in the long

run will lead to enhanced patient survival rates by allowing chemotherapy dose-intensification. Although chemotherapy dose-intensification by many is believed to enhance response and survival rates (308, 309), until now this in many cases was difficult to demonstrate. The outcome will determine whether the application of hematopoietic growth factors should be considered as supportive care, or as a vital tool in enhancing survival rates of cancer patients.

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Chapter 7

SUMMARY AND CONCLUSIONS

In this thesis both in vitro and in vivo properties of recombinant human hematopoietic growth factors are described.

In chapter 1 the effects of five different recombinant human hematopoietic growth factors (GM-CSF, G-CSF, IL-3, IL-4, and IL-6) on the in vitro proliferation of a panel of eleven human SCLC cell lines is described. The results demonstrate that the in vitro proliferation of only one cell line (GLC-20) could be stimulated by one of the hematopoietic growth factors tested (rhIL-3). RhGM-CSF, rhG-CSF, rhIL-4, rhIL-6 and the autocrine growth factors insulin and bombesin, could not further augment the rhIL-3 supported proliferation. RhIL-3 binding studies demonstrated the presence of high affinity and low affinity rhIL-3 receptors on the GLC-20 cells. Finally, it was shown that the toxic effects of adriamycin and cisplatin on the proliferation of the GLC-20 cell line could partially be abrogated in the presence of rhIL-3. These data demonstrate that in some cases hematopoietic growth factors can modulate the in vitro proliferation of non-hematopoietic tumor cells and counteract the effects of chemotherapeutic drugs.

In chapter 2 the results of a double-blind placebo controlled trial, in which thirty patients with chemotherapy related leukopenia and fever were treated with standard antibiotics and seven days of intravenously administered rhGM-CSF or placebo (2.8 μg/kg/day), are reported. The aim of this study was to investigate whether rhGM-CSF treatment resulted in an accelerated recovery of the hematopoiesis of this patient group. RhGM-CSF administration resulted in a faster percentage increase of peripheral neutrophil count after two and three days of treatment, except in patients treated with ablative chemotherapy followed by autologous bone marrow transplantation. However, in this relatively small study, rhGM-CSF did not shorten the period of fever or antibiotic administration. An increased in vivo release of tumor necrosis factor-alpha and IL-6 was not observed. These data suggest that a subpopulation of patients with chemotherapy related leukopenia and fever may benefit from rhGM-CSF treatment in view of the observed effects on the neutrophil count. In order to further evaluate our observations a multicentre trial has been initiated.

A different approach to chemotherapy-induced myelosuppression is attempting to prevent or reduce the severity of this myelosuppression by treating the patients with a hematopoietic growth factor. Chapter 3 contains the results of a placebo-controlled double-blind dose-finding trial with rhGM-CSF (1.5, 3, or 6 μg/kg s.c.) administered on days 6-12 to patients treated with chemotherapy (carboplatin-cyclophosphamide on day 1) for advanced ovarian cancer. RhGM-CSF, given on an outpatient basis, demonstrated ability to raise the mean leukocyte and

neutrophil count at all dose levels as compared with the control group. Neutrophil counts of less than $0.5x10^9$/l occurred less frequently at the 6 μg/kg/day rhGM-CSF dose level compared to the placebo-treated group. At 3 and 6 μg/kg/day rhGM-CSF also increased the mean platelet count in comparison with the placebo group. Chemotherapy dose reduction or postponement due to myelotoxicity occurred in 9/28 cycles in the placebo group versus 5/44 cycles in the rhGM-CSF group, a difference which was not significant. RhGM-CSF related toxicity consisted of local reactions at the injection site, with local skin infiltrates necessitating withdrawal from the study of two patients. Capillary leakage of ^{131}I-albumin was increased in all patients after the first chemotherapy course, but was not affected by rhGM-CSF treatment. Tumor necrosis factor-alpha and C-reactive protein serum levels increased during rhGM-CSF administration at the 6 μg dose level, but Interleukin-6 serum levels were not raised by rhGM-CSF administration. We conclude that a dose of 3 and 6 μg/kg/day rhGM-CSF reduces the severity of neutropenia and thrombocytopenia after carboplatin-cyclophosphamide at the cost of in general mild side effects.

Although our study with rhGM-CSF in ovarian cancer (chapter 3) demonstrated a positive effect on the platelet counts, results from other studies demonstrate a variable effect of rhGM-CSF on thrombopoiesis. RhIL-3 is a hematopoietic growth factor that affects the myeloid, erythroid and megakaryocytic lineage both in vitro and in vivo. In chapter 4 the results of a phase I/II trial with rhIL-3 in patients with advanced ovarian cancer eligible for treatment with chemotherapy (carboplatin-cyclophosphamide) are reported. Patients received 1, 5, 10 or 15 μg/kg/day rhIL-3 (days 5-11) in cycles 1, 3 and 5 by continuous i.v. infusion or once daily s.c. administration. In control cycles 2, 4 and 6 no rhIL-3 was administered. RhIL-3 significantly increased the recovery of leukocyte, neutrophil and platelet counts, especially at 5, 10 and 15 μg/kg rhIL-3. No difference in efficacy between s.c. and i.v. rhIL-3 treatment was found. The incidence of chemotherapy postponement for insufficient bone marrow recovery was significantly reduced in the rhIL-3 treated patients. RhIL-3 up to doses of 10 μg/kg/day could be administered without severe side effects. At 15 μg/kg/day rhIL-3 headache was dose-limiting. RhIL-3 slightly raised tumor necrosis factor alpha, C-reactive protein and serum amyloid A plasma levels, while no effect on Interleukin-6 plasma levels was observed. We conclude that rhIL-3 administered to patients on chemotherapy treatment for ovarian cancer reduces both neutropenia and thrombocytopenia and that up to 10 μg/kg/day rhIL-3 can be given safely on an outpatient basis.

Chapter 5 contains the results of a pharmacokinetic study of rhIL-3 administered s.c. and by continuous i.v. infusion (1, 5, 10 or 15 μg/kg/day) in patients treated with chemotherapy for advanced ovarian cancer as described in chapter 4. During the seven day course no plasma accumulation of s.c. administered rhIL-3 occurred. No significant difference in elimination half life times between s.c. and i.v. administered rhIL-3 could be demonstrated. In addition, the bioavailability of s.c. administered rhIL-3 was nearly 100%. Since there was no difference in hematological efficacy between the two routes of rhIL-3 administration, as

already demonstrated in chapter 4, we conclude that the s.c. route of administration appears to have no disadvantages over the i.v. route and may facilitate its clinical application.

Finally, in chapter 6 the role of hematopoietic growth factors in standard dose chemotherapy regimens and in chemotherapy dose-intensification, including ablative chemotherapy followed by bone marrow rescue is reviewed.

We conclude that the introduction of rh hematopoietic growth factors to clinical oncology and hematology has created the opportunity to ameliorate to a certain extent chemotherapy-induced myelosuppression. For both rhGM-CSF, as partially demonstrated in this thesis, and rhG-CSF there is evidence that these hematopoietic growth factors can reduce the duration of neutropenia following standard dose chemotherapy as well as after ablative chemotherapy and bone marrow transplantation. Promising as the results may be, more studies in a wide range of tumor types will have to be performed to clearly demonstrate that application of rhGMCSF or rhG-CSF will lead to a decrease in the rate of infectious complications, antibiotic use and hospitalization, and finally in an enhanced patient survival. Results may be further improved by defining the optimal dose and administration schedule of these cytokines, especially during multiple cycles of standard dose chemotherapy.

At this stage, rhIL-3 seems the first candidate for reducing chemotherapy-induced thrombocytopenia. In addition, it also stimulates neutropoiesis. However, the rhIL-3 related side effects are more outspoken compared to rhGM-CSF and rhG-CSF. It may well be that the earlier acting hematopoietic growth factors have a less favorable toxicity profile as these factors act on several cell lineages and may enhance the release of multiple cytokines, partially responsible for the side effects.

Other possible candidates for stimulation of thrombopoiesis and myelopoiesis (e.g. IL-1 and IL-6) have reached the early stages of clinical investigation. Studies in which combinations of growth factors are being used have been initiated recently, as have studies with fusion proteins.

In view of the stimulatory effect of some hematopoietic growth factors on the in vitro proliferation of tumor cell lines, certain hazards may be attached to the clinical application of these growth factors in oncology and hematology. In hematology there is evidence that hematopoietic growth factors can induce in vivo proliferation of acute myeloid leukemia tumor cells. Such an in vivo proliferation of tumor cells, induced by the administration of hematopoietic growth factors, may affect response and survival rates in patients treated with chemotherapy. Until now, there are no clinical data that demonstrate this growth factor-induced phenomenon. In contrast, growth factors may be used to stimulate proliferation of tumor cells, thus creating more vulnerable targets for chemotherapeutic drugs. However, very large clinical studies are needed to demonstrate such a possible effect of hematopoietic growth factors on response or survival rates. The same holds true for a possible anti-tumor effect of some hematopoietic growth factors.

Although hematopoietic growth factors have demonstrated ability to reduce

chemotherapy-induced myelosuppression it remains to be determined whether this will, in the long run, lead to enhanced patient survival rates by allowing chemotherapy dose-intensification. Certainly, at this stage the degree of chemotherapy dose intensification achieved by applying a single growth factor has been limited. Therefore, it seems unrealistic to expect great improvements in survival rates. A combination of hematopoietic growth factors, in which the sequence of administration reflects the in vivo mechanism of hematopoiesis may allow a more pronounced dose-intensification.

In addition, reinfusion of peripheral harvested stem cells after ablative chemotherapy may in many cases replace bone marrow reinfusion. The number of peripheral circulating stem cells, and subsequently the number of harvested cells, can be increased by administering hematopoietic growth factors. At this stage it appears that a combination of growth factors may be superior to a single growth factor. Since in the near future stem cell collecting may be mainly performed on an outpatient basis, this may facilitate chemotherapy dose-intensification in a large number of patients, as hopefully the duration of hospitalization will be shortened considerably.

The outcome of such studies will determine whether the use of hematopoietic growth factors in clinical oncology and hematology should be considered as supportive care, or as a vital tool in increasing both response and survival rates in cancer patients by allowing chemotherapy dose-intensification.

Chapter 8

SAMENVATTING EN CONCLUSIES

Eén der belangrijkste bijwerkingen van cytostaticagebruik bij kankerpatiënten is beenmergdepressie. Door het ontstaan van een tekort aan witte bloedcellen, met name de neutrofiele granulocyten, is de patiënt gevoeliger voor bacteriële infecties, terwijl een laag trombocytenaantal meer kans op het ontstaan van bloedingen geeft. Bovendien wordt beenmergdepressie beschouwd als een belangrijk obstakel bij het streven om de chemotherapie te intensiferen, teneinde betere behandelingsresultaten te verkrijgen.

Bij het merendeel van de met chemotherapie behandelde patiënten herstelt de aanmaak van bloedcellen zich in het verloop van enkele weken. Belangrijk voor de stimulering van de bloedaanmaak zijn de hematopoëtische groeifaktoren. Echter, de endogene produktie van deze eiwitten kan meestal niet voorkomen dat na chemotherapie veelal een periode van beenmergdepressie ontstaat. Met het cloneren van verschillende hematopoëtische groeifaktoren is het mogelijk geworden om in de kliniek te toetsen of exogeen toegediende groeifaktoren de duur en ernst van chemotherapie-geïnduceerde myelosuppressie kunnen reduceren.

Het doel van dit proefschrift was te onderzoeken of het gebruik van hematopoëtische groeifaktoren, zoals recombinant humaan granulocyt-makrofaag kolonie-stimulerende faktor (rhGM-CSF) en rh interleukine-3 (rhIL-3), na chemotherapie de ernst en duur van neutropenie en trombopenie kon reduceren, zonder dat dit zou leiden tot ernstige, aan deze groeifaktoren gerelateerde, bijwerkingen.

In hoofdstuk 1 worden de resultaten beschreven van een in vitro studie, waarin het effekt van vijf verschillende hematopoëtische groeifaktoren op de in vitro proliferatie van elf kleincellig longcarcinoom cellijnen werd bestudeerd. Deze studie werd uitgevoerd met het oog op mogelijk stimulerende effekten van deze faktoren op de groei van humane tumorcellen. Stimulatie van de in vitro proliferatie trad slechts op bij het toevoegen van rhIL-3 aan één cellijn, de GLC-20 lijn. Tevens werden op de GLC-20 cellijn lage- en hoge affiniteits receptoren voor IL-3 aangetoond. Geen van de andere geteste faktoren had invloed op de in vitro proliferatie van de diverse cellijnen. Bovendien werd aangetoond dat het cytotoxische effekt van adriamycine en cisplatinum op de in vitro proliferatie van de GLC-20 cellijn afnam in de aanwezigheid van rhIL-3. Deze gegevens laten zien dat in sommige gevallen hematopoëtische groeifaktoren ook de in vitro proliferatie van niet hematopoëtische tumorcellijnen kunnen beïnvloeden en in vitro het cytotoxische effekt van sommige cytostatica kan verzwakken.

Hoofdstuk 2 bevat de resultaten van een dubbelblinde, placebo gecontroleerde studie, waarin gedurende 7 dagen rhGM-CSF of placebo (2,8 μg/kg/dag) door continue intraveneuze

infusie toegediend werd aan 30 patiënten met leukopenie en koorts, ontstaan na chemotherapie. Het doel was om te bestuderen of in deze patiëntengroep rhGM-CSF veilig toegediend kon worden en of deze groep baat had bij de additionele behandeling met een groeifaktor. RhGM-CSF toediening resulteerde in een snellere percentuele toename van het aantal neutrofiele granulocyten op de tweede en derde dag van de studie, behalve bij patiënten die behandeld waren met ablatieve chemotherapie. Echter, in deze relatief kleine studie, had rhGM-CSF geen invloed op de duur van de koorts en de toediening van antibiotica. In deze groep patiënten had rhGM-CSF geen effekt op de serumspiegels van Interleukine-6 en Tumor Necrosis Faktor α. Deze gegevens suggereren dat patiënten met chemotherapie gerelateerde leukopenie en koorts baat kunnen hebben bij behandeling met een groeifaktor. Inmiddels is een landelijke ontwikkelingsgeneeskunde studie gestart om dit effekt aan te tonen.

In hoofdstuk 3 worden de resultaten beschreven van een dubbelblinde placebo gecontroleerde fase I/II studie waarin rhGM-CSF (1,5, 3 of 6 μg/kg/dag) werd gegeven aan patiënten die met chemotherapie (carboplatine en cyclofosfamide) behandeld werden voor een ovariumcarcinoom. Onder invloed van rhGM-CSF, dat poliklinisch gedurende 7 dagen subcutaan werd gegeven, beginnend 5 dagen na de toediening van chemotherapie, was het leukocyten- en neutrofielenaantal op alle dosisstappen hoger dan in de placebogroep. Ernstige neutropenie ($< 0.5 \times 10^9$/l) trad significant minder frequent op bij de met 6 μg/kg/dag rhGM-CSF behandelde patiënten dan in de placebogroep. Op de hogere dosisstappen verhoogde rhGM-CSF tevens het trombocytenaantal ten opzichte van de placebogroep. Dosisreductie of uitstel van chemotherapie op basis van myelosuppressie was noodzakelijk in 5/44 cycli in de rhGM-CSF groep versus 9/28 cycli in de placebogroep, dit verschil is echter niet significant. RhGM-CSF gerelateerde toxiciteit bestond uit lokale huidreakties op de injektieplaatsen. Lokale infiltraten noopten tot het staken van de rhGM-CSF injekties bij twee patiënten. Na de eerste chemotherapiekuur was de capillaire lekkage van ^{131}I albumine verhoogd in alle patiënten, maar dit werd niet versterkt door rhGM-CSF toediening. De serumspiegels van Tumor Necrosis Faktor α en C-reaktieve proteïne stegen licht na de hoogste dosis rhGM-CSF, maar de spiegels van Interleukine-6 stegen niet. Wij concluderen dat 3 en 6 μg/kg/dag rhGM-CSF de ernst van de neutropenie en trombocytopenie na carboplatine en cyclofosfamide reduceert ten koste van relatief milde bijverschijnselen.

Hoewel in de hierboven beschreven studie rhGM-CSF een positief effekt had op het trombocytenherstel, wordt dit effekt slechts in een klein aantal studies waargenomen. RhIL-3 is een hematopoëtische groeifaktor met een stimulerend effekt op de myeloïde, erythroïde en megakaryocytaire reeks, zowel in vitro en in vivo. In hoofdstuk 4 worden de resultaten gepresenteerd van een fase I/II studie met rhIL-3 in patiënten met ovariumcarcinoom die behandeld werden met carboplatine en cyclofosfamide. RhIL-3 (1, 5, 10 of 15 μg/kg/dag) werd gedurende 7 dagen in cyclus 1, 3 en 5 subcutaan of middels continue intraveneuze infusie toegediend, 4 dagen na het geven van chemotherapie. In de contrôlecycli 2, 4 en 6 werd geen rhIL-3 gegeven. Met name op de 5, 10 en 15 μg/kg/dag dosisstap had rhIL-3 een positief effekt

op het leukocyten-, neutrofielen-, en trombocytenherstel. Er kon geen verschil in effektiviteit tussen subcutaan en intraveneus toegediend rhIL-3 aangetoond worden. Chemotherapieuitstel op basis van onvoldoende beenmergherstel werd minder frequent waargenomen in de met rhIL-3 behandelde patiënten. De bijwerkingen van rhIL-3 bestonden uit koorts, malaise en hoofdpijn. Op 15 μg/kg/dag bleek hoofdpijn de dosislimiterende faktor. RhIL-3 verhoogde in geringe mate de plasmaspiegels van Tumor Necrosis Faktor α, C-reaktieve proteïne en serum amyloïd A, maar had geen effekt op de plasmaspiegels van Interleukine-6. Wij concluderen dat bij de cytostatische behandeling van patiënten met ovariumcarcinoom, toediening van rhIL-3 de mate van neutropenie en trombopenie reduceert.

Hoofdstuk 5 bevat de resultaten van een farmacokinetische studie met rhIL-3 in dezelfde patiëntengroep als beschreven in hoofdstuk 4. Gedurende de subcutane behandeling met rhIL-3 trad geen accumulatie in het plasma op. Er werd geen significant verschil gevonden tussen de plasmahalfwaardetijden van subcutaan en intraveneus toegediend rhIL-3. De biologische beschikbaarheid van subcutaan rhIL-3 was bijna 100%. Daar er geen verschil in effektiviteit tussen de beide toedieningswijzen van rhIL-3 bestond, concluderen wij dat subcutaan toegediend rhIL-3 geen nadelen heeft ten opzichte van intraveneus toegediend rhIL-3, hetgeen de klinische toepassing ten goede komt.

In hoofdstuk 6 wordt een overzicht gegeven van de rol van hematopoëtische groeifaktoren in zowel standaard chemotherapieschema's, als in geïntensiveerde schema's, al dan niet gevolgd door beenmergtransplantatie.

Concluderend kan worden gezegd dat met de introductie van hematopoëtische groeifaktoren in de klinische oncologie en hematologie de mogelijkheid is gecreëerd om (soms) de mate en duur van chemotherapie geïnduceerde beenmergdepressie te reduceren. Zowel rhGM-CSF, zoals beschreven in dit proefschrift, als rhG-CSF kunnen de duur van chemotherapie geïnduceerde neutropenie reduceren. Een verdere verbetering van dit resultaat kan mogelijk bereikt worden door optimalisering van de dosering en het moment van toediening van deze groeifaktoren. Hoewel de resultaten tot zover bemoedigend zijn, is nog gerandomiseerd onderzoek bij diverse tumoren vereist teneinde aan te tonen dat het gebruik hematopoëtische groeifaktoren leidt tot een afname van het aantal infekties, noodzaak tot antibiotische behandelingen en ziekenhuisopname en uiteindelijk tot een verbetering van levenskwaliteit en overlevingsduur.

Op dit moment lijkt rhIL-3 de eerst aangewezen groeifaktor bij pogingen de ernst van chemotherapie geïnduceerde trombopenie te reduceren. Bovendien stimuleert rhIL-3 de granulopoëse. Echter, de bijwerkingen van rhIL-3 lijken meer uitgesproken dan die van rhGM-CSF en rhG-CSF. Het is zeer wel mogelijk dat groeifaktoren, die op vroegere stadia van de hematopoëse aangrijpen en daardoor vaak een effekt hebben op meerdere lijnen, meer bijwerkingen geven. Gedeeltelijk kan dit gerelateerd zijn aan het vrijkomen van diverse cytokines, zoals Tumor Necrosis Faktor α en IL-6.

Gezien het in enkele gevallen optredende stimulerende effekt van groeifaktoren op de

in vitro proliferatie van tumor cellijnen, kan er mogelijk een nadeel verbonden zijn aan het in vivo gebruik van deze groeifaktoren. Echter, het is ook voorstelbaar dat groeifaktoren gebruikt kunnen worden om juist de in vivo proliferatie van tumorcellen te stimuleren teneinde de tumorcellen gevoeliger te maken voor chemotherapie. Om dit effekt van groeifaktoren op tumorrespons en overleving aan te tonen zijn echter vergelijkende studies nodig.

Hematopoëtische groeifaktoren kunnen de mate van chemotherapie geïnduceerde myelosuppressie reduceren. Echter, het is het nog onduidelijk of groeifaktoren een dusdanige chemotherapie dosisintensifikatie mogelijk maken dat ook de overlevingskansen voor kankerpatiënten zullen verbeteren. De tot nu toe bereikte mate van chemotherapie intensificatie met behulp van een enkele groeifaktor (bij benadering een verdubbeling) is nog te beperkt om een duidelijke verbetering van de overleving te verwachten. Een combinatie van groeifaktoren, waarbij de sequentie van toediening het normale in vivo mechanisme reflekteert, maakt misschien een meer uitgesproken chemotherapie dosisintensifikatie mogelijk. Reïnfusie van perifeer geoogste hematopoëtische stamcellen na ablatieve chemotherapie kan binnenkort autologe beenmergtransplantatie gaan vervangen. Het aantal perifeer circulerende stamcellen, en daarmee het geoogste aantal stamcellen, kan verhoogd worden met behulp van één of meerdere hematopoëtische groeifaktoren. Door het verkorten van de aplastische fase na chemotherapie wordt enerzijds het risiko op complicaties verkleind en kan anderzijds de chemotherapie geïntensiveerd worden. Daar het oogsten van stamcellen zich met name op een poliklinische basis af gaat spelen, kan op deze wijze mogelijk de kans op overleving bij een groot aantal patiënten vergroot worden.

Het uiteindelijke resultaat van vergelijkende studies zal moeten bepalen of het gebruik van hematopoëtische groeifaktoren in de klinische oncologie en hematologie gezien moet worden als een belangrijk onderdeel in het bereiken van verbeterde overlevingskansen van met chemotherapie behandelde kankerpatiënten.